An Investigation of the Effect of Nerve Growth Factor in the Early Stages of Neuronal Differentiation

YUNG, Him Shun

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Philosophy
in
Pharmacology

August 2007

Abstract

This project aimed to enhance our understanding of the signal transduction pathways which were regulated by neurotrophic factors such as nerve growth factor (NGF) in the early stages of neuronal differentiation, using a well-established model of rat pheochromocytoma (PC12) cells. A microarray study performed by our co-investigators at the Hong Kong University of Science and Technology showed that in the early stage of differentiation, NGF treatment (12 hours) increased cyclooxygenase-1 (COX-1) mRNA expression. Since COX-1 is required for the production of precursors for the synthesis of prostanoids from arachidonic acid, the objective of the present study was to determine if NGF had any effect on prostanoid receptors signaling in PC12 cells.

First, we characterized the prostanoid receptor expression pattern in PC12 cells grown in complete culture medium with high serum supplement (as used for general maintenance of undifferentiated cells) or cells treated with NGF (50 ng/ml) for 32 hours in low-serum medium (as used for studying the neurotrophic action of NGF) after pre-labeling cells with [^{3}H]inositol or [^{3}H]adenine. None of the prostanoid receptor agonists tested produced significant changes in [^{3}H]inositol phosphates ([^{3}H]IP) or [^{3}H]cAMP production, which suggested that no G_q- or G_s-coupled prostanoid receptors were expressed by PC12 cells. In PC12 cells grown in complete culture medium, the EP3 receptor agonists (PGE_2, sulprostone and ONO-AE-248) inhibited forskolin-stimulated [^{3}H]cAMP production, which suggested the presence of a G_i-coupled EP3 receptor. Furthermore, this EP3 agonist-mediated response was attenuated in NGF-treated cells, suggesting that EP3 receptors were expressed in PC12 cells, and that NGF might reduce EP3 receptor signaling.

As we had only studied the effect of NGF in PC12 cells at 32 hours, we made a

more extensive study of the time course effect of NGF on EP3 receptor signaling in PC12 cells and compared the responses of NGF-treated cells with control cells in low-serum medium. Since we had demonstrated that EP3 was the only EP receptor subtype found in PC12 cells, we chose PGE_2 for further study and tested the effect of NGF on PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP over 6 days. Our results suggested that the decrease of EP3 receptor-mediated responses seen at 32 hours was NGF-independent, and was due to transfer of PC12 cells from a serum-rich culture medium to the low-serum medium.

Although NGF did not affect EP3 receptor signaling in PC12 cells, we noticed that NGF could dramatically increase forskolin-stimulated AC activity after 4 to 6 days treatment. As the increased activity of AC might play an important role in the differentiation process, we therefore proceeded to study the effect of NGF on AC in PC12 cells. When NGF was removed after 2 days, there was no subsequent enhanced response of PC12 cells to forskolin. In contrast, the enhanced response to NGF was maintained on day 6 when NGF was removed after 4 days treatment. NGF was found to induce neurite extensions after 2 days treatment, and increased the proportion of PC12 cells having neurite extensions in a time-dependent manner over 6 days treatment. However, neurites were not maintained when NGF was removed after 2 or 4 days. Therefore, we concluded that cAMP production was involved in the NGF-induced initiation of neurite outgrowth in PC12 cells, and continual presence of NGF was required to maintain neurites, probably in a cAMP-independent way.

We proposed that the enhanced activation of AC by forskolin in NGF-treated PC12 cells might be due to a synergistic interaction between forskolin and an endogenous activator of a G_s-coupled GPCR. We have demonstrated the presence of G_s-coupled A_{2A} receptors in PC12 cells, and their activation might be involved in the enhanced response to forskolin due to the release of adenosine during NGF-mediated

neuronal differentiation. However, the preliminary tests suggested that adenosine deaminase (to destroy endogenously produced adenosine) or A_{2A} receptor antagonist (to block the effect of endogenously produced adenosine) appeared not to affect the enhanced effect of NGF on AC.

Taken together, our results demonstrated that the EP3 receptors were the only prostanoid receptors expressed in PC12 cells but their expression was not regulated by NGF. Furthermore, NGF was found to enhance forskolin-stimulated AC activity in PC12 cells, and this effect was persistent after 4 days NGF treatment but was not maintained when NGF was removed after 2 days. NGF stimulated neurite production of PC12 cells in a time-dependent manner, and NGF was indispensable for the maintenance of neurites. These valuable findings may allow the better understanding of the signal transduction events in the early stages of neuronal differentiation.

論文摘要

這個研究的目的在於增進我們對早期神經變異中，由神經營養因子(例如神經生長因子(NGF))所控制的信息傳遞的認識。我們利用已被充分建立的鼠嗜鉻細胞瘤(PC12)細胞作爲研究模型。我們在香港科技大學的研究夥伴對 PC12 細胞做了微陣列測試，結果顯示 12 小時的 NGF 可以增加環化加氧酶-1(COX-1)的 mRNA 水平。由於由花生四烯酸生產出前列腺素類需要 COX-1，目前的研究目標在於測定 NGF 是否影響 PC12 細胞的前列腺素類受體的信息傳遞。

首先，我們識別在對照的(生長於高血清介質)或接受 32 小時 NGF(生長於低血清介質)的 PC12 細胞中前列腺素類受體的模式。所有測試了的前列腺素類受體的激動劑都不能明顯改變[^{3}H]IP 或[^{3}H]cAMP 的生產，暗示 PC12 細胞中沒有 G_q 或 G_s 蛋白聯結的前列腺素類受體。在生長於高血清介質的 PC12 細胞中，EP3 受體的激動劑(PGE_2，sulprostone 和 ONO-AE-248)可抑制由 forskolin(一種腺苷酸環化酶(AC)活化劑)所刺激的[^{3}H]cAMP 的生產，暗示了 G_i 蛋白聯結的 EP3 受體的存在。此外，由 EP3 激動劑所傳達的反應在接受 NGF 的 PC12 細胞中會減少，暗示 EP3 受體存在於接受 NGF 的 PC12 細胞，而 NGF 可能減少 EP3 受體的信息傳遞。

由於我們只調查了 32 小時 NGF 對 PC12 細胞的效果，我們對 NGF 對 EP3 受體的信息傳遞的時間性效果做了大規模的調查，比較生長於低血清介質中的對照細胞和接受 NGF 的細胞的反應。由於我們經已展示在 PC12 細胞內只有 EP3 受體，在往後的調查我們選擇使用 PGE_2，並測試在六天的過程中，NGF 對由 PGE_2 所傳達的對 forskolin 所刺激的[^{3}H]cAMP 的生產的抑制效果。我們的結果顯示 EP3 受體反應的減少不涉及 NGF，而是因爲把培養 PC12 細胞的介質由高血清轉換至低血清的環境。

雖然 NGF 不影響 PC12 細胞內 EP3 受體的信息傳遞，我們注意到接受四天和六天 NGF 能大幅增強由 forskolin 刺激的 AC 的活性。因爲 AC 活性的增加在

變異過程中可能扮演重要角色，所以我們進一步調查在 PC12 細胞內 NGF 對 AC 的影響。當 NGF 在兩天後被去掉，PC12 細胞沒有隨後地對 forskolin 有增強反應。相反，當 NGF 在第四天被去掉，PC12 細胞在第六天仍然維持增強反應。接受兩天 NGF 可以引發 PC12 細胞的軸突伸展，在六天的過程中，NGF 可以隨時間地增加擁有軸突的 PC12 細胞的比例。可是，當 NGF 在兩天或四天後被去掉，軸突不能維持。因此，我們推斷 cAMP 的產生牽涉於由 NGF 引起 PC12 細胞的軸突生長的開始，而軸突的維持需要連續性的 NGF 的存在，並且不涉及 cAMP。

我們提議在接受 NGF 的 PC12 細胞中，forkolin 增強對 AC 的激活效應，可能是因爲 forskolin 和內源性的 G_s 蛋白聯結受體的活化劑產生協同相互作用。我們經已展示在 PC12 細胞內存在與 G_s 蛋白聯結的 A_{2A} 受體，在 NGF 引發的神經變異中可能導致腺苷的釋放，而腺苷對 A_{2A} 受體的刺激效應可能牽涉 forskolin 的增強反應。可是，初步的測試顯示腺苷脫氧酶(去除內源生產的腺苷)或 A_{2A} 受體的頡頏物(阻止內源生產的腺苷的效應)似乎不影響 NGF 對 AC 的加強效應。

總括而言，我們的結果顯示 EP3 受體是唯一存在於 PC12 細胞的前列腺素類受體在，但它們的水平不受 NGF 所調節。此外，在 PC12 細胞中，NGF 可以加強 forskolin 對 AC 的刺激效果，這效果能在接受四天 NGF 的細胞中持續，但當 NGF 在兩天後被去掉，這效果不再保持。NGF 能隨時間地刺激 PC12 細胞的軸突產生，而 NGF 對軸突的維持是必要的。以上的發現希望可以增加我們對早期神經變異的信息傳遞的認識。

Acknowledgements

I would like to express my sincerest gratitude to my supervisor, Professor Helen Wise, for all of her guidance and advice, especially on the preparation of this thesis, throughout this two years study. Also, I would like to thank the members of Department of Pharmacology for their kindly assistance and support, especially to Mr. Kevin B.S. Chow, who provided various technical supports and facilitated my experiments patiently and enthusiastically. Finally, I would like to express my deepest thanks to my friends for their motivation and understanding

This project was fully supported by a grant from the Research Grants Council of Hong Kong Special Administrative Region (HKUST3/03C). The funding of studentship from The Chinese University of Hong Kong is also acknowledged.

Publications based on work in this thesis

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Abbreviations

AC	Adenylyl cyclase
ADA	Adenosine deaminase
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived growth factor
bp	Base pairs
BSA	Bovine serum albumin
[^{3}H]cAMP	[^{3}H]cyclic adenosine monophosphate
COX	Cyclooxygenase
DAG	1,2-diacylglycerol
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNase I	Deoxyribonuclease I
DP receptor	Prostaglandin D_2 receptor
EGF	Epidermal growth factor
EP receptor	Prostaglandin E_2 receptor
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
FP receptor	Prostaglandin $F_{2\alpha}$ receptor
GPCR	G-protein coupled receptor
HCl	Hydrochloric acid
HIHS	Heat-inactivated horse serum
HRP	Horseradish peroxidase
IBMX	3-isobuty-1-methyl-xanthine

$[^3H]IP$	$[^3H]$inositol phosphates
IP receptor	Prostacyclin receptor
IP_3	Inositol 1,4,5-trisphosphate
KH_2PO_4	Potassium phosphate monobasic
LDH	Lactate dehydrogenase
LiCl	Lithium chloride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$Na_4P_2O_7$	Sodium pyrophosphate
NGF	Nerve growth factor
NO	Nitric oxide
NT	Neurotrophin
PBS	Phosphate-buffered saline
PC12 cells	Rat pheochromocytoma cells
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGs	Prostaglandins
PI3K	Phosphatidylinositol-3-OH-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTX	Pertussis toxin
PLA_2	Phospholipase A_2

PLC	Phospholipase C
PLD	Phospholipase D
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TXA_2	Thromboxane A_2
Trk	Tropomyosin-related kinase
TNF	Tumor necrosis factor
TP receptor	Thromboxane A_2 receptor

Contents

Chapter 1

Introduction

1.1 Objectives and overview of this study

The aim of this study was to examine the signal transduction pathways which were regulated by nerve growth factor (NGF) during the early stages of neuronal differentiation, and this work was in collaboration with The Hong Kong University of Science and Technology (HKUST). We choose rat pheochromocytoma (PC12) cells, which is a well-established model for studying neuronal differentiation and as they can respond to NGF and differentiate into a neuronal phenotype. When compared with stem cells, which is the ideal model for neuronal differentiation, the use of PC12 cells still has its own advantages, such as it is easier for their maintenance and numerous published literatures can be obtained for references. Perhaps, a better understanding of neuronal differentiation will inspire the discovery of novel therapeutic targets for neurodegenerative disease.

The differentiation of neuronal cells is controlled by complex networks of signal transduction pathways and gene expression, if they are not regulated appropriately, cells will be fail to become functional. Therefore, microarray technology has been used to study gene expressions which are regulated by NGF in PC12 cells. Up to now, most of the microarray data focus at a later stage of differentiation in PC12 cells

(after 4 to 5 days NGF treatment) (Lee et al., 2005). In contrast, we target on the very early stage (after 12 h NGF treatment), because the gene regulation at the early stage will determine the end stage of differentiation. We would like to perform a novel study on how the product of those genes affects NGF-induced neuronal differentiation. Our data showed that 324 genes are differentially regulated by NGF by at least 1.5 fold in PC12 cells after 12 h treatment. Of interest to us was that the cyclooxygenase-1 (COX-1) mRNA expression level was increased 4-foid by NGF. This finding is consistent to Kaplan et al., which showed that NGF increased COX-1 mRNA and protein expression in PC12 cells (Kaplan et al., 1997). Since COX-1 is involved in the synthesis of prostanoids, therefore we speculate if prostanoids and their receptors play an essential role in neuronal differentiation.

In this study, the prostanoid receptor expression in PC12 cells was determined by assaying the second messengers ([^{3}H]IP and [^{3}H]cAMP) produced after the activation of any G-protein coupled prostanoid receptor using selective receptor agonists. We demonstrated that G_i-coupled EP3 receptors were expressed in PC12 cells. After that, we investigated the time course effect of NGF on PC12 cells, and discovered that NGF could enhance forskolin-stimulated [^{3}H]cAMP production of PC12 cells. We attempted to relate this observation to the change of adenylyl cyclase (AC) expression by NGF as determined by RT-PCR. In addition, the degree of

differentiation of PC12 cells was monitored by measuring the neurofilament heavy subunit (NfH) protein expression and by assessing neurite outgrowth. Finally, we investigated if the involvement of A_{2A} receptor activity was the possible explanation for the enhancement of forskolin-stimulated [^{3}H]cAMP production in NGF-treated PC12 cells.

1.2 Rat pheochromocytoma (PC12) cells

PC12 cells were established from a transplantable rat adrenal pheochromocytoma in 1976 (Greene & Tischler, 1976). PC12 cells respond to nerve growth factor (NGF) and undergo a dramatic morphological change and acquire characteristics of sympathetic neurons. In normal growth medium, the PC12 cells have a round shape and do not extend neurite-like processes (Fig. 1.1A). However, when the PC12 cells are exposed to NGF, proliferation ceases and they differentiate into neuronal cell phenotype with long neurites (Fig.1.1B).

Because PC12 cells possess the above properties, they have become a commonly used cell model to study how neuroblasts differentiate into mature neurons under the influence of NGF (Kaplan et al., 1994; Birkaya & Aletta, 2005; Arthur et al., 2007). Also, NGF is found to act as a survival factor for PC12 cells under serum-free conditions (Greene, 1978), thus PC12 cells are also employed for

the study of the mechanisms of neuronal cell apoptosis (Shimoke et al., 2005; Kavanagh et al., 2006; Jiang et al., 2007).

1.3 Prostanoids and their receptors

Prostanoids are group of molecules comprising prostaglandins (PGs) and thromboxanes (TXs), which exert a variety of actions in various tissues and cells. For examples, prostanoids are involved in the regulation of the smooth muscle contraction and relaxation (Tilley et al., 2003; Allen et al., 2006), the glomerular and distal tubular functions in the kidney (Dominguez et al., 1984), and the secretion and motility of the gastrointestinal tract (Dey et al., 2006).

Prostanoids are synthesized by COX pathway under both physiological and pathological stimuli. COX exists as two isoforms referred to as COX-1 and COX-2 (Smith et al., 2000), and are about 60% identical with the catalytic regions being conserved (Langenbach et al., 1995). COX-1 is a constitutively expressed enzyme in many tissues, which provides various "housekeeping" functions like to strengthen gastric mucosa against injury, to regulate platelet aggregation and renal blood flow (Chan et al., 2005; Kawada et al., 2005; Peskar, 2001). In contrast, COX-2 is normally not detectable and must be induced by the pro-inflammatory agents like cytokines and lipopolysaccharide (Franciosi et al., 2005; Huang et al., 1998; Yang et

al., 2007). However, COX-2 is expressed constitutively in the central nervous system and is involved in nociceptive processes (Martin et al., 2007). COX-2 is also constitutively expressed in other tissues like in colon and kidney (Adegboyega & Ololade, 2004; Bernardini et al., 2006). In PC12 cells, COX-1 is constitutively expressed and being elevated by NGF (Kaplan et al., 1997). In contrast, COX-2 is normally not found in PC12 cells (Arenander et al., 1989).

The synthetic process of prostanoids is presented in Fig. 1.2. The starting material for the synthesis of prostanoids is arachidonic acid, which is derived from the membrane phospholipids by the action of phospholipase A_2 (PLA_2). The arachidonic acid is converted to prostaglandin G_2 (PGG_2), and then to prostaglandin H_2 (PGH_2) by the subsequent actions of COX. PGH_2 then acts as the common intermediate for the synthesis of the five prostanoids: prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), by their corresponding synthase (Smith, 1992). Prostanoids formed are then released outside of the cells facilitated by a prostaglandin transporter (Lu & Schuster, 1998). Because prostanoids like PGI_2 and TXA_2 have a very short half-life, they have to work locally at the site of their production.

Prostanoids mediate their actions through the activation of their corresponding prostanoid receptors. There are five classes of prostanoid receptors,

designated as DP for PGD_2 receptors and EP, FP, IP, and TP for PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, respectively (Narumiya et al., 1999; Breyer et al., 2001). Two receptor subtypes are found for DP (DP1 and DP2) and four subtypes for EP (EP1-4) (Breyer et al., 2001; Hirai et al., 2001).

The prostanoid receptor family belongs to the G-protein coupled receptors superfamily (GPCRs) (Narumiya et al., 1999). All GPCRs share a common structure and are integral membrane proteins with seven transmembrane helices (Muller, 2000). Each prostanoid receptor coupled with a G-protein for their signal transduction upon ligand binding. The IP, DP1, EP2 and EP4 receptors coupled principally to G_s, and their activation lead to increase of intracellular cyclic adenosine monophosphate (cAMP) through the stimulation of adenylyl cyclase (AC) (Narumiya et al., 1999). On the contrary, activation of EP3 or DP2 receptors lead to decrease of cAMP formation as it is coupled to G_i and inhibits AC activity (Hirai et al., 2001; Narumiya et al., 1999). The EP1, FP and TP receptors coupled to G_q which can activate phospholipase C (PLC) and this leads to the production of two second messengers, inositol 1,4,5-trisphosphate (IP_3) which mobilizes intracellular calcium, and 1,2-diacylglycerol (DAG) which activates protein kinase C (PKC) (Narumiya et al., 1999).

1.4 Roles of prostanoids

Prostanoids mediate various actions under different physiological conditions. Prostanoids play an important role in the central nervous system. PGD_2 is proposed to be a mediator to induce sleep in rats and other mammals (Hayaishi, 1991). Infusion of prostaglandin PGD_2 into the lateral ventricle of the brain induced an increase in the amount of non-rapid eye movement sleep, accompanied with an increase of the extracellular adenosine level in wild-type mice (Mizoguchi et al., 2001). The accumulation of adenosine in the extracellular space was found to increase the sleep propensity (Porkka-Heiskanen, 1999). However, in mice deficient in the DP receptor, the infusion of PGD_2 did not promote the accumulation of adenosine (Mizoguchi et al., 2001). Therefore, PGD_2 plays a significant role in sleep induction.

Also, prostanoids are important mediators of inflammation. Major characteristics of acute inflammation like swelling which is caused by vasodilation, and pain, are mediated by prostanoids. During inflammation, PGE_2 and PGI_2 were found to be highly expressed at the inflammatory sites (Davies et al, 1984). Together with other inflammatory mediators like histamine and bradykinin, PGE_2 and PGI_2 induce an increase in vascular permeability and hyperalgesia (Bley et al., 1998; Davies et al, 1984). Moreover, PGI_2 was found to mediate the hyperaglesic response

through the activation of IP receptors (Murata et al., 1997). Both the wild-type mice treated with indomethacin, an inhibitor of COXs (Smith et al., 1994), and the mice deficient in IP receptor, appeared to be less sensitive to the acetic acid-induced writhing test (Murata et al., 1997).

The most prominent effects of $PGF_{2\alpha}$ are on reproduction and parturition. It was reported that $PGF_{2\alpha}$ metabolite showed a linear correlation with time and number of ovulations in rabbit ovary (Schlaff et al., 1983). $PGF_{2\alpha}$ also regulates luteal cell progesterone production and synchronizes ovulation (Khan-Dawood et al., 1989; Momcilovic et al., 1998). $PGF_{2\alpha}$ is important for animals to perform parturition. The mice deficient in FP receptor have normal ovulation, fertilization and implantation as the wild-type mice, but cannot perform parturition due to the lack of labor (Sugimoto et al., 1997). Moreover, the administration of COX inhibitor which inhibited the $PGF_{2\alpha}$ synthesis led to an increase in the average length of gestation and delayed parturition (Chan et al., 1991; Lewis & Schulman, 1973).

The main function of TXA_2 is in hemostasis. The mice deficient in TP receptor have prolonged bleeding times, and their platelets appear not to aggregate after exposure to TP receptor agonists (Thomas et al., 1998). In humans, it was demonstrated that patients having a mutated TP receptor was the cause of impaired platelet aggregation (Hirata et al., 1994).

Among all the prostanoids, PGE_2 plays the most diverse actions in different parts of body, as PGE_2 can mediate its action through four EP receptor subtypes. Activation of EP1 receptors was reported to inhibit osmotic water permeability induced by arginine-vasotocin in the frog urinary bladder (Bachteeva et al., 2007), and is required for the increase of carbonate secretion in response to mucosal acidification in the rat stomach (Takeuchi et al., 2006). The EP2 receptors can induce cAMP production and Ca^{2+} release from intracellular stores of astrocytes (Di Cesare et al., 2006). In spinal cord, the EP3 receptors participate in the synthesis of nitric oxide (NO) which is essential for pain transmission (Matsumura et al., 2005). Stimulation of EP4 receptors can accelerate the regeneration of bone under bone injury in rat (Tanaka et al., 2004).

1.5 Nerve growth factor (NGF) and its receptors

The actions of NGF are via the activation of two receptors, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases, and the p75 receptor which belongs to the superfamily of the tumor necrosis factor (TNF) receptor (Ye, 2005). The Trk receptor family has three subtypes, TrkA, TrkB and TrkC, and NGF mediates different cellular effects through the activation of TrkA receptor (Rankin *et al.*, 2005). One of the functions of p75 receptor is to provide

discrimination of the binding of different neurotrophins to Trk receptors. For example, brain-derived growth factor (BDNF), neurotrophin-3 and neurotrophin-4/5, can all bind to TrkB receptors, but only BDNF can elicit functional response in the presence of p75 receptors (Bibel et al., 1999). Similarly, NT-3 normally did not induce neurite outgrowth of PC12 cells, but induced neurite outgrowth through TrkA receptors in a mutant PC12 cell line with decreased expression of p75 receptors (Benedetti et al., 1993). The p75 receptors have pro-apoptotic effects but also mediate NGF-induced survival signaling in PC12 cells (Bui et al., 2001; Yan et al., 2005).

When NGF binds to TrkA receptors, it triggers receptor dimerization and kinase activation by phosphorylation of the tyrosines in the cytoplasmic domain of TrkA receptors (Patapoutian & Reichardt, 2001). After this phosphorylation, adapter proteins are recruited for the regulation of different intracellular signaling pathways, including the Ras/extracellular-signal-regulated kinase (ERK) protein kinase, the phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase pathway and phospholipase C-γ1 (PLC-γ1) (Kaplan & Miller, 2000).

1.6 Change of gene expressions by NGF in PC12 cells

The major effects of NGF on PC12 cells are the induction of growth arrest,

accompanied by the elongation of neurites and electrical excitability (Greene & Tischler, 1976). In order to achieve these effects, NGF must alter the gene expression of PC12 cells. Different approaches have been employed to identify genes which are changed in PC12 cells by NGF treatment, including the use of cDNA microarray analysis (Lee et al., 2005), comparative expressed-sequence-tag analysis (Lee et al., 1995) and serial analysis of gene expression (Angelastro et al., 2000). The results showed that at least 1000 genes in PC12 cells were regulated by NGF.

NGF triggers cell cycle arrest in PC12 cells. Epidermal growth factor (EGF) was found to promote proliferation instead of differentiation of PC12 cells (Vaudry et al., 2002). Therefore, it is logical to see that the EGF receptors were down-regulated upon NGF treatment, which facilitates cell cycle exit and differentiation of PC12 cells (Lazarovici et al., 1997; Liu et al., 2000). NGF also down-regulated gene transcripts which are involved in DNA replication (Angelastro et al., 2000).

NGF also regulates genes which favor differentiation of PC12 cells. For example, neuritin, which was involved in axon growth (Cantallops et al., 2000), was up-regulated by NGF (Lee et al., 2005; Cappelletti et al., 2007). G_o proteins which are concentrated at the growth cones in NGF-treated PC12 cells (Zubiaur & Neer, 1993), were up-regulated in both mRNA and protein levels (Andreopoulos et al., 1995; Angelastro et al., 2000). During differentiation, neurite extensions were

observed which definitely required new synthesis of phospholipids. Phosphatidylcholine is one of the phospholipids for the formation of cell membrane, and its synthesis requires CTP:phosphocholine cytidylytransferase (CT) (Taneva et al., 2005). NGF can elevate the expression of CTβ2 isoform in PC12 cells during neurite outgrowth (Carter et al., 2003). Phospholipase D (PLD), which hydrolyses phosphatidylcholine and other phospholipids to generate phosphatidic acid, was also increased by NGF in PC12 cells, which might facilitate membrane transport to replenish plasma membranes (Hayakawa et al., 1999; Min et al., 2001).

In summary, differentiation of PC12 cells triggered by NGF is a complex process, and is controlled by regulation of multiple genes. Although with the support of modern techniques for the quick analysis of changes of gene expression induced by NGF, relatively few have been evaluated with respect to their functional significance. Therefore, there is still a long way to go to fully understand the NGF-induced differentiation of PC12 cells.

1.7 Signaling pathways involved in NGF-induced differentiation of PC12 cells

The ERK is activated by growth factors and is involved in cell proliferation or differentiation (Marshall, 1995). The cellular action of ERK depends on the duration of its activation. It has been reported that NGF could induce a sustained activation of

ERK which triggered differentiation, while the EGF only induced a transient activation of ERK kinase to stimulate proliferation in PC12 cells (Dikic et al., 1994; Traverse et al., 1994). On the other hand, in PC12 mutant cells where NGF can only induce transient ERK activation, there was no morphological change upon NGF treatment (Yaka et al., 1998). These findings suggested that a sustained activation of ERK was required for the differentiation process in the PC12 cells. Besides the Ras/ERK pathway, the PI3K/Akt pathway is also involved in differentiation of PC12 cells. NGF activates the PI3K/Akt pathway, which is particularly important for promoting survival under a wide variety of circumstances (Brunet et al, 2001; Wu & Wong, 2005). However, the PI3K/Akt pathway also participates in the initiation and maintenance of neurite outgrowth triggered by NGF in PC12 cells (Jackson et al., 1996; Kobayashi et al., 1997; Kita et al., 1998). In order to become fully functional, PC12 cells treated with NGF must have ability to synthesize and store neurotransmitter. NGF induced the expression of tyrosine hydroxylase, which is the rate-limiting enzyme for the synthesis of catecholamines, and is dependent on Ras/ERK pathway (Suzuki et al., 2004). In contrast, the induction of gene expression responsible for the synthesis of acetylcholine was mainly regulated by the PI3K/Akt pathway (Madziar et al., 2005). As a result, the differentiation of PC12 cells is controlled by multiple pathways, and their combinatory effect may determine the

ultimate fate of these cells.

1.8 Classification of adenylyl cyclases

AC is the enzyme which catalyzes the conversion of ATP to cAMP, and cAMP is an important second messenger in eukaryotic signal transduction. In mammalian cells, there are two major types of AC, the transmembrane AC (tmAC) and the soluble AC (sAC).

There are nine tmAC isoforms (AC1 to AC9) and their catalytic activities are regulated by GPCRs (Hanoune & Defer, 2001). All of the tmAC isoforms except AC9 can be activated by forskolin (Simonds, 1999). These nine isoforms can be classified into four groups: Group I AC isoforms include AC1, AC3 and AC8, which are stimulated by Ca^{2+} and calmodulin, Group II AC isoforms include AC2, AC4 and AC7, which are stimulated by $G_{\beta\gamma}$ and PKC phosphorylation, Group III AC isoforms include AC5 and AC6, which are inhibited by PKA, and Group IV isoform only contain AC9, which is inhibited by Ca^{2+} and calcineurin (Paterson et al., 2000; Chang et al., 2003a). Unlike the tmAC, sAC is not regulated by GPCRs and is insensitive to forskolin (Buck et al., 1999; Litvin et al, 2003). The activity of sAC is regulated by bicarbonate (Chen et al., 2000) and calcium (Litvin et al., 2003).

The increase of intracellular cAMP level is involved for the stimulation of

differentiation of PC12 cells, because cAMP activates ERK in PC12 cells (Frodin et al., 1994; Young et al., 1994; Erhardt et al., 1995; Vossler et al., 1997). In addition, the analog of cAMP, dibutyryl cAMP, can initiate neurite outgrowth by itself in PC12 cells (Gunning et al., 1981b; Heidemann et al., 1985; Hayakawa et al., 1999). Therefore, AC under regulation by various stimuli is related to the differentiation of PC12 cells.

1.9 Methods to study differentiation of PC12 cells

NGF triggers neurite outgrowth of PC12 cells, therefore it is a direct method to quantify the degree of differentiation by neurite assay, which is a typical assessment performed by counting the proportion of cells expressing neurites of length more than certain cell diameter or by direct measurement of neurite length (Perry et al., 2002; Attiah et al., 2003; Cappelletti et al., 2007). However, this method is not easy to perform when we have to analyze large amount of samples as it is labor-intensive, and the assessment of neurites is dependent on the observer, so it is not a fully objective measurements. As a result, it is desirable to develop alternative assessment on differentiation of PC12 cells, such as the measurement of neuronal markers or metabolic enzymes which are obviously regulated by NGF during differentiation. Several protein expressions were regarded as the markers of differentiation, such as

neurofilament proteins and axonal growth associated protein 43 (GAP-43), and their expression level is used as a reflection of the degree of differentiation (Lindenbaum et al., 1987; Edsall et al., 1997; Das et al., 2004; Liu et al., 2006). In addition, measurement of enzyme activity which is changed by NGF has also been employed. NGF was reported to suppress the synthesis of lactate dehydrogenase (LDH), while increased the activity of NADH-dehydrogenase in PC12 cells (Calissano et al., 1985; Cattaneo et al., 1985; Rhodes et al., 1989). The activity of LDH and NADH-dehydrogenase was increased and decreased, respectively, by NGF in a dose-dependent manner in PC12 cells (Ohuchi et al., 2002).

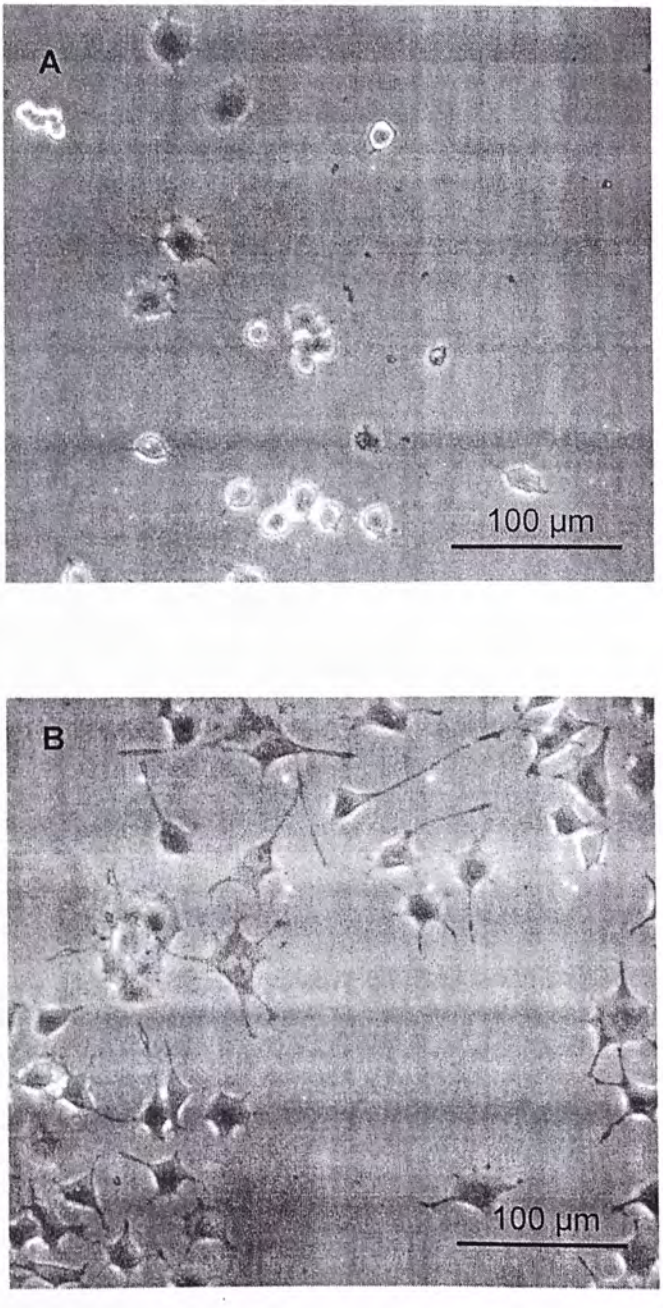


Fig. 1.1. Cell morphology of PC12 cells. (A) PC12 cells maintained in complete culture medium. (B) PC12 cells treated with 50 ng/ml of NGF in low-serum medium for 6 days.

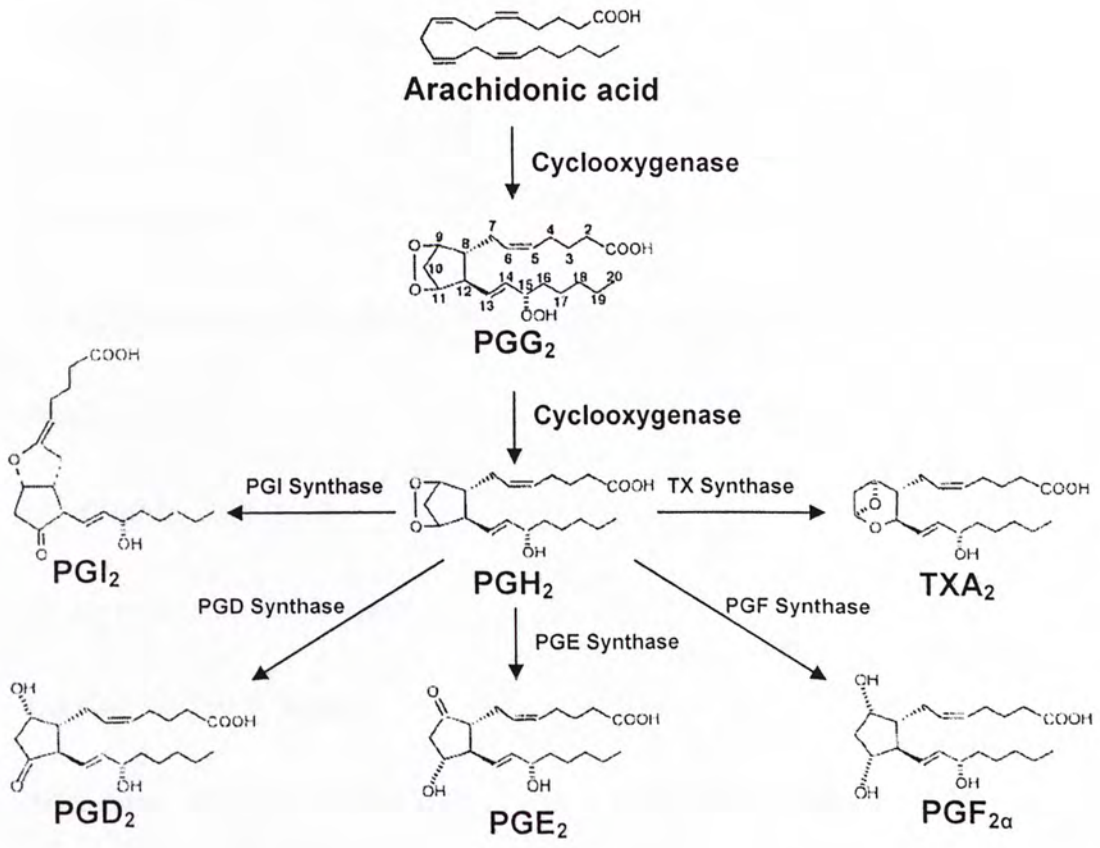


Fig. 1.2. Biosynthesis of prostanoids. The arachidonic acid is converted to PGG_2 and then to PGH_2 catalyzed by COX. Then, the PGH_2 acts as the common intermediate for the synthesis of five prostanoids, the PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$ and TXA_2 by their corresponding synthetic enzymes. This figure is taken from Narimuya et al., (1999).

Chapter 2

Materials and methods

2.1 Materials

8-[^{3}H]-adenine (specific activity 29.0 Ci/mmol) was purchased from Amersham and stored at 4 ℃.

1,4-dithiothreitol (USB)

Acetic acid (Ridedel-de Haen)

Acrylamide (30%, Sigma)

Adenosine 5'-triphosphate (ATP, Sigma) was dissolved in distilled water and freshly prepared before assay.

Adenosine deaminase from bovine spleen (Sigma)

Ammonia solution (25%, BDH)

Ammonium formate (Standard)

Ammonium persulfate (APS, Bio-Rad) was prepared as 10% (w/v) solution by dissolving 0.5 g powder in 5 ml distilled water, aliquoted and stored at -20 ℃.

Anti-neurofilament heavy subunit antibody (Covance)

Alumina (neutral type WN-3, Sigma)

Bovine serum albumin (BSA, Sigma)

Bradykinin (Sigma) was prepared as a 10 mM stock in 5% acetic acid and stored at -20 ℃.

Bromophenol blue (Sigma)

n-Butanol (Sigma)

CGS21680 (Sigma) was prepared as a 10 mM stock in DMSO and stored at -20 ℃.

Cicaprost was a gift from Schering which was prepared as a 1 mM stock in DMSO and stored at -20 ℃.

Chloroform (Sigma)

Complete protease inhibitor (Roche) was prepared as 25X stock by dissolving 1 tablet in 2 ml distilled water, being aliquoted and stored at -20 ℃.

Dimethyl sulphoxide (DMSO, Sigma)

DNase I (Invitrogen)

dNTP Mix (Invitrogen)

Dowex AG1-X8 (100-200 mesh, formate form) was purchased from Bio-Rad.

Dowex AF50W-X4 (200-400 mesh, hydrogen form) was purchased from Bio-Rad.

Dulbecco's modified Eagle's medium (DMEM, Gibco) was dissolved in distilled water with the addition of 3.7 g $NaHCO_3$ per litre of medium. The medium was adjusted to pH 7.2, then filter sterilized and stored at 4 ℃.

ECL™ anti-mouse IgG, horseradish peroxidase-linked (Amersham)

ECL chemiluminescence kit (Amersham)

EDTA (Ethylenediamine-tetraacetic acid, Sigma)

Ethanol (absolute, Merck)

Ethidium bromide (Sigma)

Expand High Fidelity^{PLUS} PCR System (Roche)

Fetal bovine serum (FBS, Gibco)

Formic acid (BDH)

Forskolin (Sigma) was dissolved in DMSO at 10 mM and stored at -20 ℃.

Glucose (Sigma)

Glycerol (Sigma)

Glycine (Sigma)

Heat-inactivated horse serum (HIHS, Gibco)

HEPES (N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, Sigma)

Hydrochloric acid (HCl, BDH)

Hybond nitrocellulose membranes (Amersham)

IBMX (3-isobuty-1-methyl-xanthine, Sigma) was prepared as 100 mM stock in DMSO and stored at -20 ℃.

Imidazole (Sigma)

Inositol, myo-[2-^{3}H(N)] (specific activity 18.5 Ci/mmol) was purchased from NEN Life Science Products, Inc. and stored at 4 ℃.

Isopropanol (BDH)

Lithium chloride (Sigma) was prepared as 1 M solution in distilled water and stored at 4 ℃.

Micro BCA Protein Assay Kit (Pierce)

M-MLV reverse transcriptase (Invitrogen)

NGF (mouse nerve growth factor 2.5s, grade II) was purchased from Alomone Labs (Israel). 25 μg lyophilized powder was dissolved in 0.5 ml sterile DMEM to a concentration of 50 μg/ml, aliquoted and stored at -70 ℃.

Non-fat milk powder (Nestle)

Oligo d(T)20 primer (Invitrogen)

ONO-AE-248 was a gift from ONO Pharmaceuticals Co. which was prepared as 10 mM solution in DMSO and stored at -20 ℃.

ONO-AE1-259 was a gift from ONO Pharmaceuticals Co. which was prepared as 10 mM solution in DMSO, aliquoted and stored at -20 ℃.

ONO-AE1-329 was a gift from ONO Pharmaceuticals Co. which was prepared as 10 mM solution in DMSO, aliquoted and stored at -20 ℃.

ONO-AE3-240 was a gift from ONO Pharmaceuticals Co. which was prepared as 10 mM solution in DMSO, aliquoted and stored at -70 ℃.

ONO-DI-004 was a gift from ONO Pharmaceuticals Co. which was prepared as 10 mM solution in DMSO and stored at -20 ℃.

OptiPhase 'Hi-safe' 3 (Pharmacia Biotech)

Penicillin/Streptomycin solution (Gibco)

Potassium chloride (KCl, Sigma)

Potassium phosphate monobasic (KH_2PO_4, Sigma)

Primer pairs (Invitrogen)

Prostaglandin D_2 (PGD_2, Cayman) was dissolved in ethanol at 10 mM and stored at -20 ℃.

Prostaglandin E_2 (PGE_2, Sigma) was dissolved in ethanol at 10 mM and stored at -20 ℃.

Prostaglandin $F_{2\alpha}$ ($PG\ F_{2\alpha}$, Sigma) was dissolved in DMSO at 10 mM and stored at -20 ℃.

Polyprep column (Bio-Rad)

Rat tail Collagen, Type I (BD Bioscience) was prepared in 0.02 N acetic acid at 4.05 mg/ml and stored at 4 ℃.

RNaseOUT^{TM} ribonuclease inhibitor (Invitrogen)

Sodium bicarbonate ($NaHCO_3$, Sigma)

Sodium chloride (NaCl, Sigma)

Sodium dodecyl sulfate (SDS, Bio-Rad)

Sodium hydroxide (NaOH, Sigma)

Sodium phosphate dibasic (Na_2HPO_4, Sigma)

Sodium pyrophosphate ($Na_4P_2O_7$, Sigma)

Sulprostone (Caymen) was dissolved in DMSO at 10 mM and stored at -20 ℃.

TEMED (Sigma)

Trichloroacetic acid (TCA, Sigma)

Triton X-100 (Sigma)

Trizma base (Sigma)

TRIZOL reagent (Invitrogen)

Trypan blue (Blue)

Trypsin/EDTA (Sigma)

U46619 (Sigma) was dissolved in ethanol at 2.85 mM and stored at -20 ℃.

ZM241385 (Tocris) was dissolved in DMSO at 5 mM and stored at -20 ℃.

Common laboratory reagents not listed (ACS grade) were purchased from Sigma. All disposable culture plates were supplied by Nunc.

2.2 Cell culture medium and buffers

Complete culture medium was prepared by mixing 25 ml FBS (5% v/v), 50 ml HIHS (10% v/v) and 425 ml DMEM in 500 ml bottle and stored at 4 ℃. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added before use.

Low-serum medium was prepared by mixing HIHS (1% v/v) with DMEM. Penicillin (100 units/ml) and streptomycin (100 μg/ml) was added before use.

Phosphate-buffered saline (PBS) was prepared with the following ACS reagents supplied by Sigma: KCl (2.7 mM), KH_2PO_4 (1.4 mM), NaCl (137 mM) and Na_2HPO_4 (8.1 mM) dissolved in distilled water. The PBS was adjusted to pH 7.5, filter sterilized and stored at 4 ℃.

2.3 Buffers and solutions for assay of [^{3}H]inositol phosphates ([^{3}H]IP) production

HEPES-buffered saline (HBS) was prepared with the following ACS reagents supplied by Sigma: HEPES (15 mM), NaCl (140 mM), KCl (4.7 mM), $CaCl_2$ (2.2 mM), $MgCl_2$ (1.2 mM) and KH_2PO_4 (1.2 mM) dissolved in distilled water. The HBS was adjusted to pH 7.4, filter sterilized and stored at 4 °C. Glucose (11 mM) was added immediately before use.

Formic acid (1 M) was prepared by diluting 37.73 ml 26.5 M stock in 1 L distilled water and stored at room temperature.

Formic acid (20 mM) was prepared by mixing 20 ml 1 M formic acid with distilled water and made up to 1 L and stored at room temperature.

Ammonia solution (0.05%) was prepared by dissolving 2 ml 25% ammonia solution in distilled water and made up to 1 L. It was stored at room temperature.

Ammonium formate (4 M) was prepared by dissolving 252.54 g ammonium formate powder in distilled water and made up to 1 L. It was stored at room temperature.

Ammonium formate (2 M)/formic acid (0.1 M) was prepared by dissolving 252.24 g ammonium formate powder with 200 ml 1 M formic acid and distilled water and made up to 2 L. It was stored at room temperature.

Ammonium formate (40 mM) /formic acid (0.1 M) was prepared by dissolving 5.04 g ammonium formate with 200 ml 1 M formic acid and distilled water and made up to 2 L. It was stored at room temperature.

Lithium chloride (1 M) was prepared by dissolving 424 mg lithium chloride powder in 10 ml distilled water and stored at 4 ℃. It was diluted in HBS to a final concentration of 20 mM.

2.4 Buffers and solutions for assay of [^{3}H]cAMP production

HEPES-buffered saline (HBS) was the same as used in [^{3}H]-IP production assay.

Imidazole solution (0.5 M) was prepared by dissolving 104.5 g imidazole powder and 27 g NaOH in distilled water and made up to 2 L. The solution was adjusted to pH 7.5 and stored at room temperature.

Imidazole solution (0.1 M) was prepared by mixing 200 ml 0.5 M imidazole solution with distilled water and made up to 1 L when required.

Hydrochloric acid (4 M) was prepared by diluting 330 ml 12.2 M stock with 670 ml distilled water and stored at room temperature.

Hydrochloric acid (1 M) was prepared by mixing 250 ml 4 M HCl with 750 ml distilled water and stored at room temperature.

Trichloroacetic acid (5%) was prepared by dissolving 25 g TCA in 500 ml distilled water and stored at 4 ℃.

IBMX was prepared as 100 mM stock in DMSO and stored at -20 ℃. It was diluted in HBS to a final concentration of 1 mM.

2.5 Buffers and solutions for Western blotting

Lysis buffer was prepared with the following reagents supplied by Sigma: Trizma base (50 mM), NaCl (100 mM), EDTA (5 mM), $Na_4P_2O_7$ (67 mM), and Triton X-100 (0.01%) in distilled water. The solution was adjusted to pH 7.5 and made up to 1 L. It was stored at 4 ℃. Appropriate amount of 25X complete protease inhibitor was added to give a 1X working concentration just before use.

Stacking buffer was prepared by dissolving 30.2 g Trizma base in 400 ml distilled water. The solution was adjusted to pH 6.8 with HCl and made up to 500 ml and stored at 4 ℃.

Separating buffer was prepared by dissolving 90.8 g Trizma base in 400 ml distilled water. The solution was adjusted to pH 8.8 with HCl and stored at 4 ℃.

SDS solution (10%) was prepared by dissolving 10 g SDS in distilled water. The volume was made up to 100 ml and stored at room temperature.

Running buffer (10X) and transfer buffer (10X) was prepared by dissolving 60 g Trizma base and 288 g glycine in distilled water. The volume was made up to 2 L and stored at room temperature.

Running buffer (1X) was prepared by mixing 200 ml 10X running buffer and 20 ml 10% SDS solution in distilled water. The volume was made up to 2 L and stored at room temperature.

Transfer buffer (1X) was prepared by mixing 600 ml 10X transfer buffer and 900 ml absolute ethanol in distilled water. The volume was made up to 6 L and stored at room temperature.

Sample buffer (6X) was prepared by mixing 7 ml stacking buffer, 1 g SDS, 0.93 g 1,4-dithiothreitol, 3 ml glycerol and 1.2 mg bromophenol blue in 10 ml distilled water. It was aliquoted and stored at -20 °C.

Ammonium persulfate (APS) solution (10%) was prepared by dissolving 0.5 g APS in 5 ml distilled water. It was aliquoted and stored at -20 ℃.

n-Butanol (water-saturated) was prepared by mixing 300 ml n-butanol with 100 ml distilled water. The top clear layer was used after it separated into 2 phases.

Tris buffer saline (TBST) (10X) was prepared by dissolving 121.11 g Trizma base and 175.32 g NaCl in 600 ml of distilled water. The solution was adjusted to pH 7.5, and then 10 ml of Tween 20 was added. The volume was made up to 2 L and stored at room temperature.

Tris buffer saline (TBST) (1X) was prepared by diluting 100 ml 10X TBST with 900 ml distilled water and stored at room temperature.

Non-fat milk solution (5%) was prepared by dissolving 1 g non-fat milk powder in 20 ml 1X TBST. It was stored at 4 ℃.

BSA solution (5%) was prepared by dissolving 1 g BSA powder in 20 ml 1X TBST. It was stored at 4 ℃.

2.6 Methods

2.6.1 Maintenance of PC12 cells

PC12 cells were cultured in 10 ml complete culture medium in a 100 mm culture dish in a humidified atmosphere of 5% CO_2/95% air at 37 ℃. Cells were passaged once a week at approximately 80% confluency, and the medium was renewed every two to three days. When subculturing the cells, the medium was removed and cells were washed with 3 ml PBS. The PBS was then removed and 2 ml culture medium was added. Cells were detached by forceful aspiration and the cell suspension was transferred to a 15 ml centrifuge tube. Cells were collected by centrifuging at 140 × g for 5 min at room temperature. The supernatant was removed and the cell pellet was resuspended in culture medium. The cell number was counted using a hemocytometer. When counting cells, 10 µl cell suspension was mixed with 10 µl trypan blue solution, and the cell viability was over 90%. About 3×10^6 cells were then plated in a new culture dish with the addition of 10 ml complete culture medium.

2.6.2 General culture condition of PC12 cells for NGF treatment

The culture plates were coated with collagen (0.2 mg/ml, diluted the stock with sterile water) for 10 min (use 2 ml or 0.5 ml collagen solution per well for 6-well or 24-well culture plates, respectively). Then, wells were emptied by aspiration and the plates were air-dried before use. PC12 cells were plated in collagen-coated in 2 ml or 0.5 ml complete culture medium in 6-well or 24-well culture plates, respectively, and incubated for 24 h in a humidified atmosphere of 5% CO_2/95% air at 37 ℃. The medium was removed and the cells were washed four times with PBS. Then, low-serum medium was added for another 24 h in a humidified atmosphere of 5% CO_2/95% air at 37 ℃. Then, DMEM or NGF (50 ng/ml) was added with day of NGF addition defined as Day 0. Culture medium and drugs were replaced every two days, as required. For the study of NGF withdrawal effect, the low-serum medium containing NGF was removed and replaced with plain low-serum medium.

2.6.3 Determination of phospholipase C activity in PC12 cells

2.6.3.1 Principle of assay

The PLC family consists of 13 isoforms and are activated by either G_q proteins or by transmembrane receptors with tyrosine kinase activity. PLC-β possesses a long C-terminal extension which is required for activation by G_q. PLC metabolizes the

membrane inositol phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to produce two second messengers, inositol 1,4,5-trisphosphate (IP_3) which mobilizes intracellular calcium, and 1,2-diacylglycerol (DAG) which activates protein kinase C (PKC) (Dean & Beaven, 1989). IP_3 was rapidly broken down which caused difficulty on its estimation. However, lithium ions were found to prevent the breakdown of IP_3 by inhibiting inositol polyphosphate-1-phosphatase (Inhorn and Majerus, 1987) and inositol monophosphatase (Fauroux & Freeman, 1999). As a result, the presence of lithium ions caused a substantial accumulation of total inositol phosphates, including the inositol trisphosphate, bisphosphate and in particular of inositol monophosphate (Berridge et al., 1982).

PC12 cells were pre-labeled with [^{3}H]inositol which was incorporated into phosphatidylinositol lipids like PIP_2. The total [^{3}H]inositol phosphates ([^{3}H]IP) production after stimulation by agonist were separated using Dowex column chromatography. The assay of [^{3}H]IP fraction in the presence of lithium ions provides an accurate estimation of the degree of receptor activation of PLC by agonist (Chow et al., 2001).

2.6.3.2 Column preparation

Dowex AG1-X8 resin was washed twice with distilled water. Resin suspension

(2 ml) was then transferred into polyprep columns. The columns were reusable and washed with 5 ml 2 M ammonium formate/0.1 M formic acid, 2 ml distilled water and then 2 ml 0.05% ammonia solution before use.

2.6.3.3 Measurement of [^{3}H]IP production

PC12 cells (3×10^5 cells/well) were cultured in 0.5 ml medium in 24-well plates as described in section 2.2. Cells were pre-labeled with [^{3}H]inositol (2 μCi/ml; 1 μCi/well) for 32 h before the assay of [^{3}H]IP production. On the day of assay, the medium was aspirated and the cells were washed twice with 0.5 ml HBS followed by 10 min incubation at 37 ℃ in HBS containing LiCl (20 mM) to prevent the breakdown of [^{3}H]IP_3. The buffer was removed and replaced with 0.5 ml fresh HBS (with 20 mM LiCl) followed by the addition of 5 μl vehicle or test compounds for 1 h at 37 ℃ in a water bath. The reaction was stopped by aspiration of the buffer and addition of 0.75 ml ice-cold formic acid (20 mM). The plates were left on ice for at least 1 h for the extraction of acid soluble total [^{3}H]inositol phospholipids fraction.

[^{3}H]IP was separated from the total [^{3}H]inositol phospholipids by column chromatography (Chow et al., 2001). The columns were placed over 20 ml scintillation vials and the entire 750 μl of formic acid extracts were loaded onto the columns, and immediately followed by the addition of 3 ml 0.05% ammonia solution

to elute the total [^{3}H]inositol phospholipids into the scintillation vials. The extracts were then mixed thoroughly with 7.5 ml of scintillant (OptiPhase 'Hi-safe' 3).

The columns were then placed over a waste container and washed with 4 ml 40 mM ammonium formate/0.1 M formic acid. The columns were then placed over another set of 20 ml scintillation vials. The [^{3}H]IP fraction was eluted by 5 ml 2 M ammonium formate/0.1 M formic acid. The extracts were then mixed thoroughly with 10 ml scintillant (OptiPhase 'Hi-safe' 3).

Blank samples (formic acid) were run through the columns in parallel to the test samples for the determination of the background count of the columns, and this value was subtracted from all the test values. The radioactivities of all the samples were measured by liquid scintillation counting (Packard LS2900TR).

2.6.3.4 Data analysis

Each experiment was performed in triplicate. The [^{3}H]IP production is expressed as [^{3}H]IP/([^{3}H]IP + [^{3}H]inositol phospholipids) × 100. The [^{3}H]IP production of the control (cells treated with vehicle only) is defined as 100%, and the agonist-stimulated [^{3}H]IP production is normalized against the control and expressed as percentage of control.

2.6.4 Determination of adenylyl cyclase activity in PC12 cells

2.6.4.1 Principle of assay

AC is the enzyme which converts ATP to cAMP, and the increase of cAMP level is required for the activation of protein kinase A (PKA). The activity of AC is determined by metabolic labeling of the cellular pool of ATP using. [^{3}H]adenine. The [^{3}H]cAMP produced in the presence of IBMX (a phosphodiesterase inhibitor to prevent the breakdown of [^{3}H]cAMP) after stimulation by agonist was separated from other radio-labeled materials using Dowex column chromatography. The [^{3}H]cAMP production in the presence of IBMX reflects the activity of AC (Chow et al., 2001).

2.6.4.2 Column preparation

Dowex AG50W-X4 resin (30 g) was mixed with 60 ml 1M HCl. Equal volume of resin suspension was transferred into polyprep columns (up to1 ml mark of the columns) . The columns were washed with 4 ml 1 M HCl followed by 10 ml distilled water. The columns were reusable and washed with 10 ml distilled water before use.

Neutral alumina (0.6 g) was put into each polyprep column and the columns were washed with 8 ml 0.1 M imidazole solution. The columns were reusable and washed with 8 ml 0.1 imidazole solution before use.

2.6.4.3 Measurement of [^{3}H]cAMP production

PC12 cells (3×10^5 cells/well) were cultured in 0.5 ml medium in 24-well plates as described in section 2.2. Cells were pre-labeled with [^{3}H]adenine (2 μCi/ml; 1 μCi/well) for 24 h before the assay of [^{3}H]cAMP production. On the day of assay, the medium was aspirated and the cells were washed twice with 0.5 ml HBS. The buffer was removed and replaced with 0.5 ml fresh HBS plus 1 mM IBMX followed by the addition of 5 μl vehicle or test compound for 30 min at 37 °C in a water bath. For the study involving the use of inhibitor or antagonist, cells were pre-incubated with inhibitor or antagonist for 30 min before the addition of test compound. The reaction was stopped by aspiration of the buffer and addition of 1 ml ice-cold 5% TCA with 1 mM ATP. The plates were left on ice for at least 1 h for the extraction of [^{3}H]cAMP and other radiolabeled materials.

[^{3}H]cAMP was separated from the total [^{3}H]adenine nucleotides ([^{3}H]AXP) by column chromatography (Chow et al., 2001). The Dowex columns were then placed over 20 ml scintillation vials and the entire 1 ml acid extracts were loaded on to the columns, followed by 3 ml distilled water to elute the [^{3}H]AXP into the scintillation vials. The extracts were mixed thoroughly with 7 ml of scintillant (OptiPhase 'Hi-safe' 3).

The Dowex columns were then placed over the alumina columns and washed

with 10 ml distilled water. When water had completely run through both set of columns, the alumina columns were placed over another set of 20 ml scintillation vials and [^{3}H]cAMP was eluted by 6 ml 0.1 M imidazole solution. The extracts were mixed thoroughly with 10 ml of scintillant (OptiPhase 'Hi-safe' 3).

Blank samples (5% TCA) were run through the columns in parallel to the test samples for the determination of the background count of the columns, and this value was subtracted from all the test values. The radioactivities of all the samples were measured by liquid scintillation counting (Packard LS2900TR).

2.6.4.4 Data analysis

Each experiment was preformed in duplicate or triplicate. The [^{3}H]cAMP production is expressed as % conversion, i.e. [^{3}H]cAMP/([^{3}H]cAMP + [^{3}H]AXP) x 100. The [^{3}H]cAMP production of the control (cells treated with vehicle only) is defined as 100%, and the agonist-stimulated [^{3}H]cAMP production is normalized against the control and expressed as percentage of control.

2.6.5 Determination of neurofilament protein expression in PC12 cells by Western blotting

PC12 cells (3×10^5 cells/well) were cultured in 2 ml medium in 6-well plates as described in section 2.2. At the end of drug treatment, medium was aspirated and the cells were washed with 1 ml ice-cold PBS. Then, 0.2 ml lysis buffer was added to each well and cells were lysed for 60 min on ice. The cell lysates were then centrifuged at 15,000 × g for 15 min at 4 ℃. The supernatant was collected and protein concentrations were determined using a Micro BCA Protein Assay Kit with bovine serum albumin (BSA) as standard.

Equal amount of protein (20 μg) from the cell lysates were loaded into 6% SDS-PAGE gel. After separation of proteins by electrophoresis at 100V for 90 min, proteins were transferred onto Hybond nitrocellulose membranes (Amersham) at constant current of 350A for 90 min. Then, the membranes were blocked with 5% BSA for 30 min at room temperature and incubated with primary antibody (anti-neurofilament antibody, 1:2000) in 3 ml 5% BSA overnight at 4 ℃. Immunodetection was done with horseradish peroxidase (HRP)-linked anti-mouse antibody (1:2000) as the secondary antibody and developed with an ECL chemiluminescence kit. The band intensity was quantified by using the computer program Scion Image (Scion Corporation, Frederick, USA).

2.6.6 Determination of adenylyl cyclase isoform expression in PC12 cells by reverse transcriptase-polymerase chain reaction (RT-PCR)

2.6.6.1 Isolation of total cellular RNA

PC12 cells (3×10^5 cells/well) were cultured in 0.5 ml medium in 24-well plates as described in section 2.2. Cells were detached by aspiration with PBS and collected by centrifugation. Cells were lysed in 1 ml TRIZOL reagent with repetitive pipetting (~15-20 times). The lysed samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml chloroform was added to the samples which were shaken vigorously by hand for 15 sec. The samples were incubated for about 3 min at room temperature and centrifuged at 12,000 × g for 15 min at 4 ℃. Following centrifugation, the upper aqueous phase containing RNA was transferred to another microcentrifuge tube, and RNA precipitated using 0.5 ml isopropanol and incubated for 10 min at room temperature. The samples were centrifuged at 12,000 × g for 10 min at 4 ℃, and the supernatants were removed. The gel-like RNA pellet was washed once with 1 ml 75% ethanol, and centrifuged at 7,500 × g for 5 min at 4 ℃. The supernatants were then removed and the remaining pellet was partially dried in air for about 5 min. Finally, the RNA pellet was dissolved in 10 µl DEPC-treated water and stored at -70 ℃. RNA extracted from adult rat (male, 150-200 g) cerebral cortex tissue (0.1g) was

used as the positive control, and the extraction process was identical to above.

The concentration and purity of the RNA extract was determined by measuring its absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). A pure preparation of RNA extract would have an A_{260}/A_{280} ratio range from 1.5 to 2.0.

RNA concentration (μg/μl) = A_{260} × 40 × dilution factor / 1000

An A_{260} of 1 corresponds to approximately 40 μg/ml for single-stranded RNA.

2.6.6.2 Synthesis of first strand cDNA by reverse transcription (RT)

The extracted RNA was used to synthesize the first strand cDNA using M-MLV reverse transcriptase. In order to remove any contamination of genomic DNA, 1 μg RNA extract was incubated with DNase I (0.5 units) for 15 min at room temperature. Then, the DNase I was inactivated by the addition of EDTA (1.25 mM) and heated for 10 min at 65 ℃ in a thermocycler (Stratagene).

The RNA samples were then incubated with dNTP mix and oligo d(T)20 primer for 5 min at 65 ℃ in the thermocycler, and then quickly chilled on ice. After the RT buffer (1X), DTT (0.1 μM) and RNaseOUT™ Recombinant Ribonuclease inhibitor (20 U) was added, the samples were incubated for 2 min at 37 ℃. MLV reverse transcriptase (100 U) was then added and incubated for 50 min at 37 ℃. Finally, the reaction was inactivated by heating the sample for 15 min at 70 ℃. The cDNA

samples were stored at -20 ℃ before use.

2.6.6.3 Polymerase Chain Reaction (PCR)

For each sample, 1 μl cDNA was mixed with forward and reverse primers of the targeted sequences (0.4 μM of each primer) and these sequences were amplified with *Taq* polymerase (0.5 U). The primer sequences used were based on published literature (Chang et al., 2003a), and the sequences are listed in Table 2.1. GAPDH gene was included as the internal control. For PCR reaction, the reaction mixtures in PCR tubes were put into a thermocycler programmed as follows: initial denaturation at 95 ℃ for 5 min, followed by 28 cycles with denaturation at 95 ℃ for 30 sec, annealing at 58 ℃ for 30 sec and elongation at 72 ℃ for 30 sec. In order to complete the reaction, the mixture was incubated at 72 ℃ for 7 min (Chang et al., 2003a). The PCR products were then stored at 4 ℃ before agarose gel electrophoresis.

2.6.6.4 Agarose gel electrophoresis

The PCR products were resolved by 2.2% agarose gel electrophoresis. Briefly, 10 μl PCR products were mixed with 2 μl 6X gel loading buffer. Then, the mixture was loaded into the wells of a 2.2% agarose gel containing ethidium bromide, with electrophoresis at a constant voltage of 100 V for about 1 h. The PCR products were

visualized under UV exposure and the images were captured, and band intensities were analyzed with Scion Image.

2.6.7 Neurite quantification

PC12 cells (5×10^4 cells/well) were cultured in 2 ml medium in 6-well plates as described in section 2.2. At specific time points, cells were analyzed manually by observation using microscope under 100 X magnification, or in another way, 5 images of each well were captured under 100X magnification using an image capturing system (DS Camera Control Unit DS-L1, Nikon). The images were quantified with Scion Image in a way that the observer was unaware of the treatment group. One hundred cells per well were scored for neurites equal to or greater than that of the cell body diameter.

2.6.8 Trypan blue exclusion test

The cell viability of PC12 cells during the assay of [^{3}H]cAMP production was determined by the trypan blue exclusion test. PC12 cells (3×10^5 cells/well) were cultured in 0.5 ml medium in 24-well plates as described in section 2.2. After the drug treatment, assay buffer was removed by aspiration, and cells were incubated with 0.2 ml trypsin for 1 min at room temperature. After that, 0.25 ml HBS was

added to dilute the trypsin. A total volume of 0.45 ml cell suspension from each well was transferred to a microcentrifuge tube containing 0.25 ml serum-containing medium (to neutralize the activity of trypsin). Another 0.5 ml HBS was added to wash out any remaining cells from the wells.

Cells were collected by centrifugation at 140 × g for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in 0.2 ml HBS. Before counting the cells, 10 µl cell suspension was mixed with 10 µl trypan blue. Viable cells will have a clear cytoplasm, while dead cells will have a blue cytoplasm. The experiment was performed in triplicate and each sample was counted in duplicate. The percentage of viable cells was defined as: counts of cells with clear cytoplasm/total cell counts × 100%.

Gene Name	Primers	PCR product size (bp)
AC2	Forward: 5'-GGG AAG ATT AGT ACC ACG GAT-3' Reverse: 5'-AGG AGA AGC CAA GGA TGG ACG-3'	334
AC6	Forward: 5'-TGC TGC TGG TCA CCG TGC TCA T-3' Reverse: 5'-GGA CGC TAA GCA GTA GAT CAT AGT TGT CAA-3'	495
AC8	Forward: 5'-CAG TCT GGG CCT GAG GAA ATT-3' Reverse: 5'-AAG TCA GGT TCT TCA AGG GTA-3'	478
GAPDH	Forward: 5'-TAT GAC AAC TCCCTC AAG AT-3' Reverse: 5'-AGA TCC ACA ACG GAT ACA TT-3'	317

Table 2.1. Primers sequences for the PCR and the expected PCR product sizes.

Chapter 3

Results

3.1 Characterization of prostanoid receptor expression in PC12 cells

3.1.1 Study of the presence of G_q-coupled prostanoid receptors

PLC-β is activated by G_q and leads to IP_3 formation. Therefore, the presence of any G_q-coupled receptors in both undifferentiated and NGF-treated PC12 cells can be determined by measuring the increase of [^{3}H]IP production after incubation with various agonists, after pre-labelling cells with [^{3}H]inositol. PGD_2, PGE_2 and $PGF_{2\alpha}$ were used to activate DP, EP and FP receptors, respectively. In contrast, PGI_2 and TXA_2 are chemically unstable (Narumiya et al., 1999), therefore, their synthetic mimetics, cicaprost and U46619, were employed to test for the presence of IP and TP receptors, respectively. Another five agonists were used to distinguish the EP receptor subtypes. Sulprostone is selective for the mouse EP3 receptors (K_i =0.6 nM), but also has considerable effect on the mouse EP1 receptors (K_i =21 nM) (Narumiya et al., 1999), and ONO-DI-004 is selective for the mouse EP1 receptors (K_i =18 nM), ONO-AEI-259 is selective for the mouse EP2 receptors (K_i =3 nM), ONO-AE-248 is selective for the mouse EP3 receptors (K_i =7.5 nM), ONO-AEI-329 is selective for the mouse EP4 receptors (K_i =9.7 nM), and the K_i values of these four EP receptor agonists to others EP receptor subtypes are at least 100-fold greater than that of their

principal EP receptor (Suzawa et al., 2000). The binding affinities of these agonists on their corresponding receptors which were represented by their inhibitory constant (K_i) values were at nanomolar concentrations. However, the agonist concentrations initially tested were much greater than their K_i values in order to achieve maximum receptor activation.

In undifferentiated PC12 cells cultured in complete culture medium, none of the prostanoid receptor agonists tested increased [^{3}H]IP production (Fig. 3.1). In order to prove that the inability of these prostanoid receptor agonists to stimulate [^{3}H]IP production was not due to the failure of the experimental setup, bradykinin and ATP were included as positive controls. Both bradykinin and ATP have been shown to increase [^{3}H]IP production through the activation of the G_q-coupled bradykinin (B_2) receptors and P2Y receptors in PC12 cells, respectively (Graness et al., 1997; Moskvina et al., 2003). The results show that bradykinin and ATP significantly increased [^{3}H]IP production in undifferentiated PC12 cells, to a level of 582 ± 38% and 397 ± 17% compared with control group, respectively (Fig. 3.1).

In PC12 cells treated with NGF for 32 h in low-serum medium, prostanoid receptor agonists were also unable to increase [^{3}H]IP production (Fig. 3.2). In contrast, bradykinin and ATP significantly increased [^{3}H]IP production in NGF-treated PC12 cells, to a level of 1412 ± 38% and 644 ± 107% compared with

control group, respectively (Fig. 3.2). The [^{3}H]IP production induced by bradykinin in NGF-treated PC12 cells was significantly greater than that in undifferentiated PC12 cells (P<0.001). The ATP-induced [^{3}H]IP production in NGF-treated PC12 cells was also greater than that in undifferentiated PC12 cells, but this effect was not statistically significant.

These results suggest that G_q-coupled prostanoid receptors were not expressed in either undifferentiated or NGF-treated PC12 cells. However, other G_q-coupled receptors such as B_2 and P2Y receptors were found in PC12 cells.

3.1.2 Study of the presence of G_s-coupled prostanoid receptors

All tmAC are activated by G_s and subsequently catalyze the conversion of ATP to cAMP. Therefore, the presence of any G_s-coupled receptors in either undifferentiated or NGF-treated PC12 cells can be determined by measuring the increase of [^{3}H]cAMP production after incubation with various agonists, after pre-labelling cells with [^{3}H]adenine.

In undifferentiated PC12 cells cultured in complete culture medium, none of the prostanoid receptor agonists tested increased [^{3}H]cAMP production (Fig. 3.3). PC12 cells express G_s-coupled adenosine A_{2A} receptors (Florio et al., 1999; Nie et al., 1999), therefore, the selective A_{2A} receptor agonist, CGS21680, was also tested as a

positive control. CGS21680 greatly increased the [^{3}H]cAMP production by 388 ± 86% conversion compared with control group (Fig. 3.3).

In PC12 cells treated with NGF for 32 h in low-serum medium, prostanoid receptor agonists were also unable to increase [^{3}H]cAMP production (Fig. 3.4), while CGS21680 significantly increased [^{3}H]cAMP production in NGF-treated PC12 cells by 298 ± 32% conversion compared with control group, respectively (Fig. 3.4). Although the [^{3}H]cAMP production induced by CGS21680 was slightly lowered in NGF-treated PC12 cells, this difference was not statistically significant when compared with undifferentiated PC12 cells.

These results suggest that G_s-coupled prostanoid receptors were not expressed in either undifferentiated or NGF-treated PC12 cells. However, other G_s-coupled receptors such as A_{2A} receptors were found in PC12 cells.

3.1.3 Study of the presence of G_i-coupled prostanoid receptors

The tmAC (except AC9) are directly activated by forskolin which dramatically increases the cellular cAMP level in different cell types (Insel & Ostrom, 2003). On the contrary, cAMP level is lowered by G_i as it exerts an inhibitory effect on adenylyl cyclase. Therefore, the forskolin-stimulated cAMP production will be inhibited in the presence of activated G_i-coupled receptors.

In undifferentiated PC12 cells cultured in complete culture medium, PGE_2 significantly inhibited the forskolin-stimulated [^{3}H]cAMP production by 49.4 ± 3.2% (P<0.01), suggesting the presence of G_i-coupled EP3 receptors (Fig. 3.5). Two EP3 receptor agonists, sulprostone and ONO-AE-248, also inhibited forskolin-stimulated [^{3}H]cAMP production by 29.8 ± 2.5% and 14.7 ± 1.1%, respectively (P<0.01) (Fig. 3.5). $PGF_{2\alpha}$ also significantly inhibited forskolin-stimulated [^{3}H]cAMP production by 23.7 ± 4.1% (P<0.01) (Fig. 3.5).

In PC12 cells treated with NGF for 32 h in low-serum medium, only PGE_2 and sulprostone could still generate a significant inhibition of forskolin-stimulated [^{3}H]cAMP production (P<0.01) (Fig. 3.6). Moreover, the inhibition exerted by PGE_2, $PGF_{2\alpha}$, ONO-AE-248 and sulprostone in NGF-treated PC12 cells were all lower than that in untreated cells (Fig. 3.7), but this was only statistically significant for PGE_2 (P<0.05). CGS21680 did not show any inhibition of the forskolin response in either cell types, confirming that activation of a classical G_s-coupled receptor does not inhibit forskolin-stimulated cAMP production.

These results suggest that G_i-coupled EP3 receptors were expressed in both undifferentiated and NGF-treated PC12 cells. PC12 cells might also express G_i-coupled FP receptors, or $PGF_{2\alpha}$ may be acting on EP3 receptors when tested here at 1 μM (K_i =3 and 75 nM, for FP and EP3 receptor, respectively) (Narumiya et al.,

1999).

3.1.4 Further proof of EP3 expression in PC12 cells

In order to confirm that the results of the assay of [^{3}H]cAMP production in PC12 cells was not affected by the endogenous production of prostanoids, the basal and the forskolin-stimulated [^{3}H]cAMP production was determined in the presence of indomethacin, a non-selective COX inhibitor (Kawai, 1998). Results suggest that neither basal nor forskolin-stimulated [^{3}H]cAMP production was affected by indomethacin in undifferentiated PC12 cells (Fig. 3.8).

Although PGE_2 activates all four EP receptor subtypes, PGE_2 was selected for the later study as EP3 receptors were the only possible subtype expressed in PC12 cells from the results of cAMP assay, and PGE_2 exerted the greatest inhibitory response on forskolin-stimulated [^{3}H]cAMP production among all the EP3 receptor agonists studied.

The standard way to prove the existence of certain receptors is to perform a functional assay in the presence of the selective receptor antagonist. ONO-AE3-240 was employed as the selective EP3 receptor antagonist, with a K_i value of 0.23 nM to EP3 receptor, and the K_i values of this compound to other three EP receptor subtypes are at least 250-fold greater (Amano et al., 2003). ONO-AE3-240 inhibited the

PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production in PC12 cells in a dose-dependent manner (Fig. 3.9), and within the tested range, ONO-AE3-240 did not have any significant influence on basal or forskolin-stimulated [^{3}H]cAMP production (Table 3.1). The PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production was almost completely blocked by ONO-AE3-240 at 10^{-7} M. These findings support our conclusion that PC12 cells express G_i-coupled EP3 receptors. For additional confirmation, we could further prove the expression of G_i-coupled EP3 receptors in PC12 cells by the addition of pertussis toxin (PTX), which can ADP-ribosylate and inactivate G_i protein (Wu & Wong, 2005). Under these conditions, the EP3 agonists should no longer inhibit forskolin-stimulated [^{3}H]cAMP production.

3.1.5 Discussion

In this part of study, we aimed to characterize the prostanoid receptor expression profile in PC12 cells by measuring the effect of different prostanoid receptor agonists on [^{3}H]IP or [^{3}H]cAMP production. Our results suggest that PC12 cells do not appear to express DP, EP1, EP2, EP4, IP or TP receptors, as their selective agonists did not show any significant effect on [^{3}H]IP or [^{3}H]cAMP production. In contrast, PC12 cells definitely express EP3 receptor, and the evidence for FP receptor is more

controversial and will be discussed later.

In undifferentiated PC12 cells, PGE_2 exerted greatest inhibition of forskolin-stimulated [^{3}H]cAMP production among all the tested compounds, and the two other EP3 receptors agonist, sulprostone and ONO-AE-248, also inhibited forskolin-stimulated [^{3}H]cAMP production. Moreover, the EP3 receptor antagonist, ONO-AE3-240, completely blocked the PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production. These findings indicate the presence of G_i-coupled EP3 receptor in PC12 cells, which is consistent with another study showing abundant expression of mRNA for EP3 receptor in PC12 celis (Kitanaka et al., 1996).

Although sulprostone has a considerable ability to activate EP1 receptors (Narumiya et al., 1999), it was logical to exclude the possible existence of G_i-coupled EP1 receptor. First, the principal transduction of EP1 receptors is through G_q, however, the selective EP1 receptor agonist, ONO-DI-004, did not stimulate [^{3}H]IP production, which did not support the presence of EP1 receptor in PC12 cells. Second, ONO-DI-004 only exerted a negligible effect on forskolin-stimulated [^{3}H]cAMP production, therefore, it was unlikely that there was EP1 receptors coupled to G_i. $PGF_{2\alpha}$ also significantly inhibited forskolin-stimulated [^{3}H]cAMP production, but it was possibly due to the cross activation of EP3 receptor by 1 μM

concentration (Narumiya et al., 1999). The principal transduction of FP receptor is through G_q, but $PGF_{2\alpha}$ did not stimulate [^{3}H]IP production, therefore, PC12 cells were not likely to express FP receptors. Although a G_i-coupled FP receptor has been characterized in the rabbit kidney (Hebert et al., 2005), the study of mRNA expression suggested an absence of FP mRNA in PC12 cells (Kitanaka et al., 1996). As a result, the existence of a G_i-coupled FP receptor in PC12 cells was questionable. It is necessary to perform an antagonist study using ONO-AE3-240 to clarify any involvement of EP3 receptor response mediated by $PGF_{2\alpha}$, or using the FP receptor antagonist (e.g. AL-8810) to clarify the presence of FP receptor in PC12 cells.

NGF treatment induces changes of receptor responses in PC12 cells. NGF caused a prominent increase in bradykinin-induced [^{3}H]IP production in PC12 cells after 32 h treatment. This increase of the bradykinin receptor response was consistent with the study showing that the number of B_2 receptors was increased by NGF in PC12 cells (Bush et al., 1991). The ATP-induced [^{3}H]IP production was also slightly increased after NGF treatment, however, it was not statistically significant. NGF was found to suppress the mRNA expression of $P2Y_6$ and $P2Y_{11}$ receptors in PC12 cells after 72 h treatment, but had no effect on other P2Y receptor subtypes (Arthur et al., 2007). Therefore, it was not expected that there would be a significant change of the P2Y receptors expression and their responses after NGF treatment which is much

shorter than 72 h in our experiments. The [^{3}H]cAMP production mediated through the activation of A_{2A} receptors by CGS21680 was slightly decreased by NGF treatment in PC12 cells, although it was not statistically significant. Our microarray data also suggested an 8.6-fold decrease of A_{2A} mRNA expression after 12 h NGF treatment. The slight decrease of the A_{2A} receptor response was possibly due to the NGF-induced down-regulation of A_{2A} receptor expression (Arslan et al., 1997).

So far, we have characterized the prostanoid receptor expression profile in NGF-treated PC12 cells, and provided strong evidence to support the presence of G_i-coupled EP3 receptors in PC12 cells. The decrease of the EP3 receptor response in PC12 cells might be due to the effects of NGF alone or the transfer of cells from high serum state to a lower serum state, and subsequent experiments were performed to find the explanation (see section 3.2).

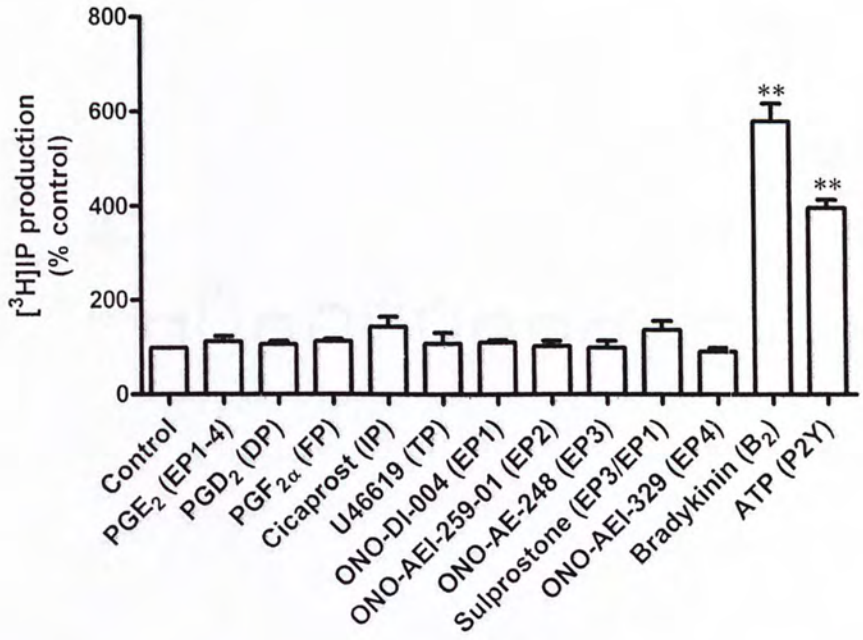


Fig. 3.1. Undifferentiated PC12 cells do not express G_q-coupled prostanoid receptors. PC12 cells were cultured in complete culture medium for 48 h. After pre-labelling with [^{3}H]inositol, PC12 cells were incubated with different prostanoid receptor agonists (1 μM), or bradykinin (10 μM) or ATP (100 μM) as the positive controls, for 1 h. Agonist-stimulated [^{3}H]IP production has been normalized against the control within each experiment which was defined as 100%. Control [^{3}H]IP production was 3.22 ± 0.62. Results represent mean ± S.E.M. of three independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

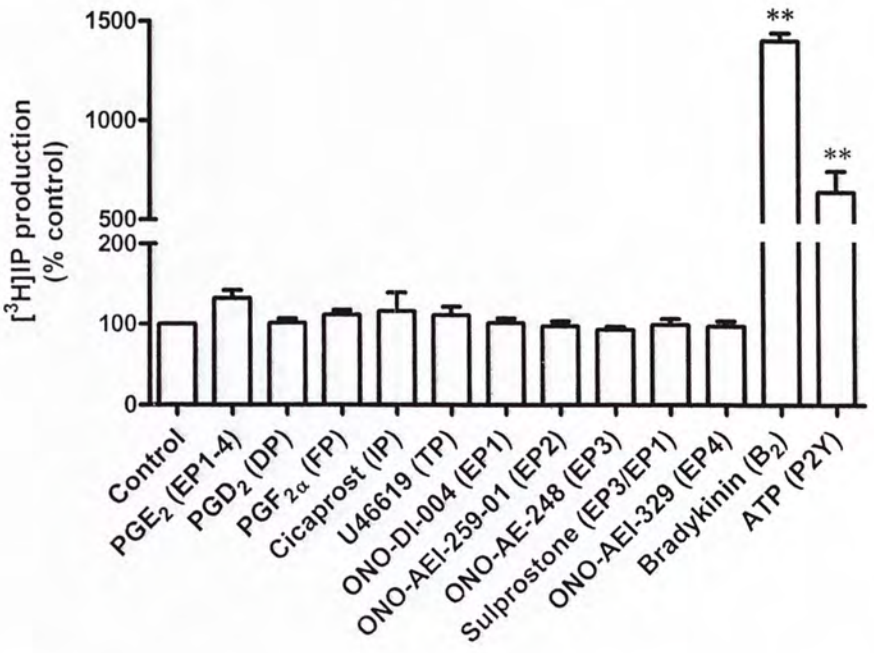


Fig. 3.2. NGF-treated PC12 cells do not express G_q-coupled prostanoid receptors. PC12 cells were treated with NGF (50 ng/ml) for 32 h in low-serum medium. After pre-labelling with [^{3}H]inositol, PC12 cells were incubated with different prostanoid receptor agonists (1 μM), or bradykinin (10 μM) or ATP (100 μM) as the positive controls, for 1 h. Agonist-stimulated [^{3}H]IP production has been normalized against the control within each experiment which was defined as 100%. Control [^{3}H]IP production was 3.27 ± 0.31. Results represent mean ± S.E.M. of three independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

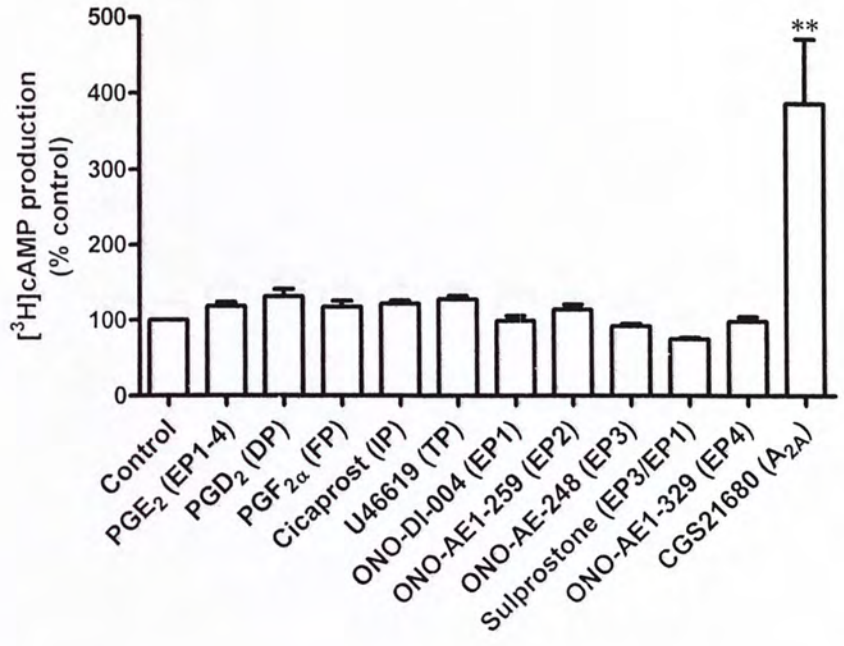


Fig. 3.3. Undifferentiated PC12 cells do not express G_s-coupled prostanoid receptors. PC12 cells were cultured in complete culture medium for 48 h. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with different prostanoid receptor agonists (1 μM) or the adenosine A_{2A} receptor agonist, CGS21680 (1 μM), as the positive control, for 30 min. Agonist-stimulated [^{3}H]cAMP production has been normalized against the control within each experiment which was defined as 100%. Control [^{3}H]cAMP production was 0.070 ± 0.005% conversion. Results represent mean ± S.E.M. of four independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

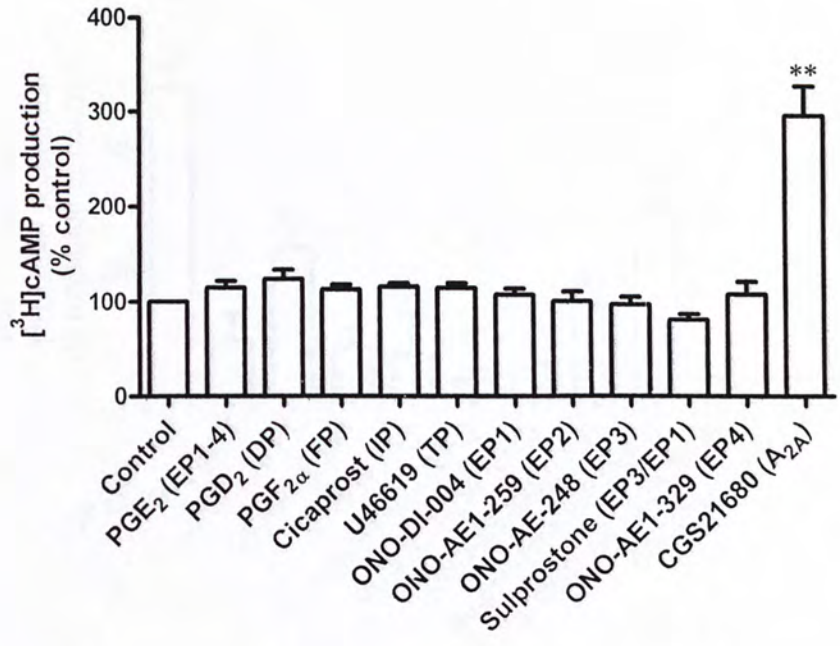


Fig. 3.4. NGF-treated PC12 cells do not express G_s-coupled prostanoid receptors.

PC12 cells were treated with NGF (50 ng/ml) for 32 h in low-serum medium. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with different prostanoid receptor agonists (1 μM) or the adenosine A_{2A} receptor agonist, CGS21680 (1 μM), as the positive control, for 30 min. Agonist-stimulated [^{3}H]cAMP production has been normalized against the control within each experiment which was defined as 100%. Control [^{3}H]cAMP production was 0.093 ± 0.011% conversion. Results represent mean ± S.E.M. of four independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

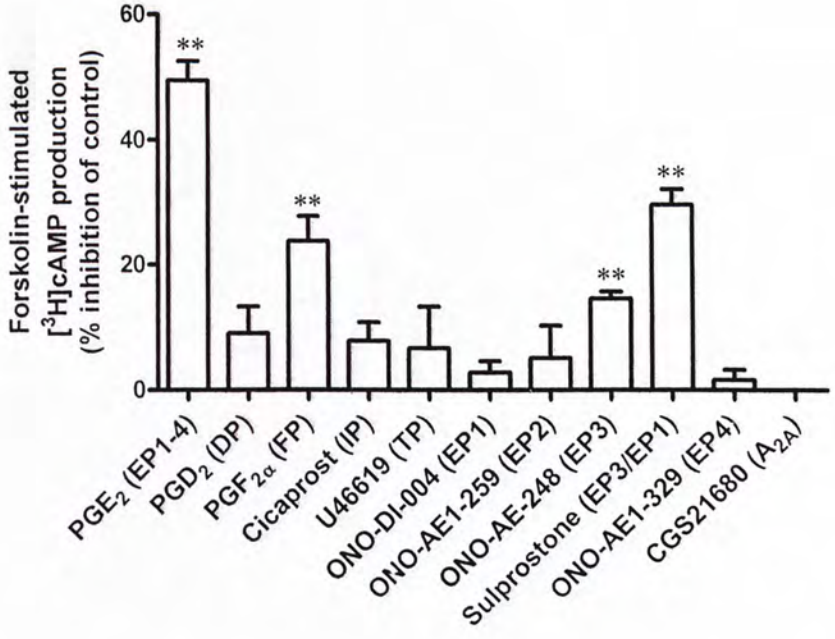


Fig. 3.5. Undifferentiated PC12 cells express G_i-coupled prostanoid receptors. PC12 cells were cultured in complete culture medium for 48 h. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with different prostanoid receptor agonists (1 μM) or CGS21680 (1 μM), in the presence of forskolin (1 μM), for 30 min. The forskolin-stimulated [^{3}H]cAMP production of control was defined as 100%. Basal and forskolin-stimulated [^{3}H]cAMP production was 0.070 ± 0.005% and 0.426 ± 0.059% conversion, respectively. Results represent mean ± S.E.M. of four independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

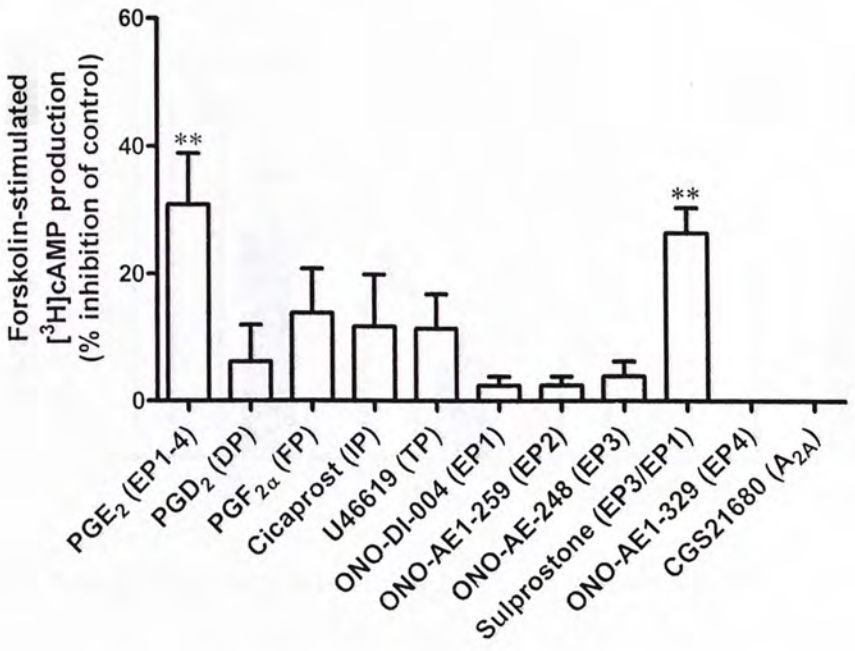


Fig. 3.6. NGF-treated PC12 cells express G_i-coupled prostanoid receptors. PC12 cells were treated with NGF (50 ng/ml) for 32 h in low-serum medium. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with different prostanoid receptor agonists (1 μM) or CGS21680 (1 μM), in the presence of forskolin (1 μM), for 30 min. The forskolin-stimulated [^{3}H]cAMP production of control was defined as 100%. Basal and forskolin-stimulated [^{3}H]cAMP production was 0.093 ± 0.011% and 0.442 ± 0.052% conversion, respectively. Results represent mean ± S.E.M. of four independent experiments performed in triplicate. (**P<0.01, one-way ANOVA with Dunnett's post test.)

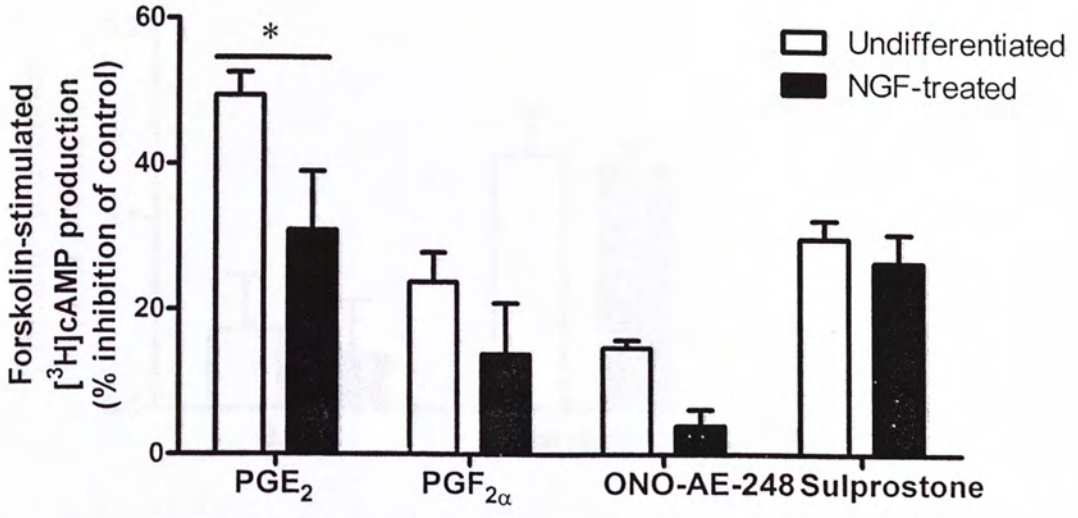


Fig. 3.7. Comparison of inhibition of forskolin-stimulated [^{3}H]cAMP production mediated by PGE_2, $PGF_{2\alpha}$, ONO-AE-248 or sulprostone in undifferentiated and NGF-treated PC12 cells. PC12 cells were either cultured in complete culture medium for 48 h (undifferentiated) or treated with NGF (50 ng/ml) for 32 h in low-serum medium. Values are mean ± S.E.M. of four independent experiments. (**P<0.01, two-way ANOVA with Bonferroni post test.)

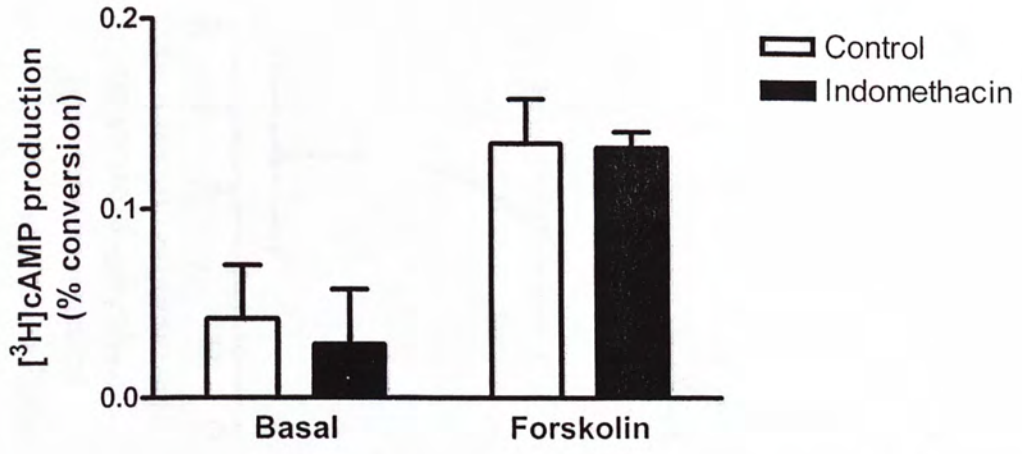


Fig. 3.8. Indomethacin (COX inhibitor) did not affect the basal or forskolin-stimulated [^{3}H]cAMP production in untreated PC12 cells. PC12 cells were cultured in complete culture medium for 48 h. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with HBS or forskolin (1 μM) in the presence or absence of indomethacin (3 μM) for 30 min. Results represent mean ± S.D., from one experiment performed in duplicate.

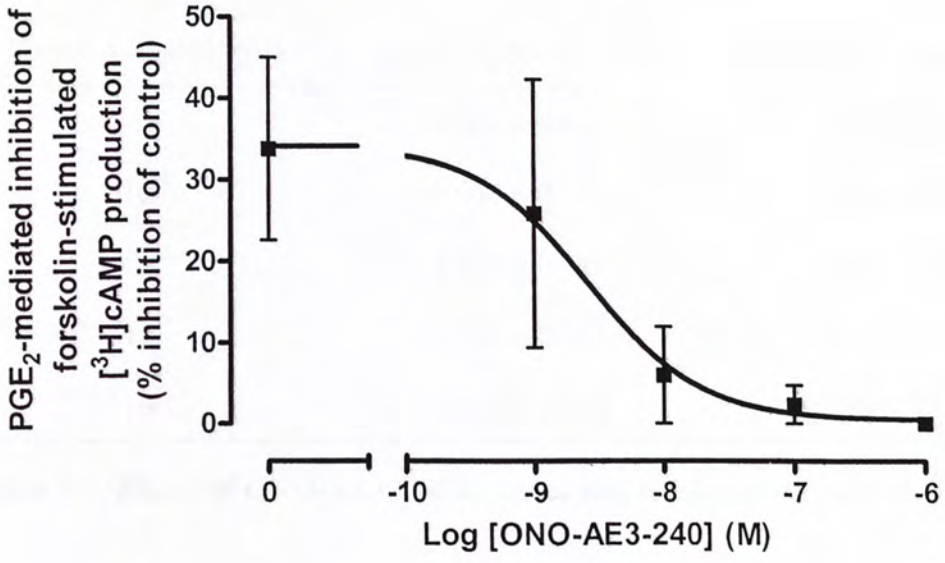


Fig. 3.9. The effect of ONO-AE3-240 (EP3 receptor antagonist) on PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production in untreated PC12 cells. PC12 cells were cultured in low-serum medium and the assay was performed on Day 0. Cells were pre-treated with different concentrations of ONO-AE3-240 for 30 min, then incubated with HBS or PGE_2 (1 μM) in the presence or absence of forskolin (1 μM) for another 30 min. Each data point represent mean ± S.E.M. of two to three independent experiments performed in duplicate or triplicate. Basal and forskolin-stimulated [^{3}H]cAMP production at various concentration of ONO-AE3-240 are listed in Table 3.1.

	[^{3}H]cAMP production	
[ONO-AE3-240] (M)	Basal (% conversion)	Forskolin (% conversion)
0	0.072 ± 0.015	0.272 ± 0.198
10^{-9}	0.064 ± 0.001	0.228 ± 0.171
10^{-8}	0.050 ± 0.026	0.240 ± 0.139
10^{-7}	0.095 ± 0.053	0.167 ± 0.070
10^{-6}	0.055 ± 0.033	0.174 ± 0.061

Table 3.1. Effect of ONO-AE3-240 on basal and forskolin-stimulated [^{3}H]cAMP production of undifferentiated PC12 cells. Values of basal and forskolin-stimulated [^{3}H]cAMP production at various concentration of ONO-AE3-240 in Fig. 3.9 are listed. ONO-AE3-240 did not affect the basal or forskolin-stimulated [^{3}H]cAMP production within the tested range. (Data analyzed with one-way ANOVA with Bonferroni post test.)

3.2 Time course effect of NGF on PC12 cells

3.2.1 Effect of NGF on PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production

In section 3.1, we have demonstrated that G_i-coupled EP3 receptors were expressed in both undifferentiated and NGF-treated PC12 cells, as the EP3 receptor agonists were able to suppress the forskolin-stimulated [^{3}H]cAMP production. In addition, we noted that the inhibitory effect exerted by these EP3 agonists appeared to be lower in PC12 cells treated with NGF. We therefore made a hypothesis that the decrease of EP3 receptor response was caused by the NGF treatment. However, undifferentiated PC12 cells were normally maintained in complete culture medium which was supplemented with high content of serum, while PC12 cells were transferred from high serum-containing medium to a medium with reduced serum supply during NGF treatment. Therefore, the change of the culturing conditions might also be responsible for the change of EP3 receptor response in PC12 cells. In this part of study, we aim to determine the time-dependent effect of NGF on PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production in PC12 cells maintained in culture medium with either high or low serum supply over 6 days treatment.

In PC12 cells cultured in complete culture medium, the PGE_2-mediated

inhibition of forskolin-stimulated [^{3}H]cAMP production of control group was increased from about 20% to 60% after 12 h, then decreased gradually to day 2 and maintained steady to day 4, and the inhibition was almost completely lost on day 6 (Fig. 3.10). For the NGF-treated group, the PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production was unchanged from day 0 to day 4, and the levels of inhibition were slightly lower than the control group during this period (Fig. 3.10). However, an unexpected increase of the PGE_2-mediated inhibition of forskolin response was observed on day 6 in the NGF-treated group (Fig. 3.10). Unfortunately, the variation of the data was large and it is difficult to draw any clear conclusion showing that NGF is responsible for the decrease of PGE_2-mediated inhibition on forskolin response. The PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production was calculated using four values, they were the [^{3}H]cAMP production values of HBS or PGE_2 in the presence or absence of forskolin, variation of any of the four values would cause large variation of the calculated inhibitory response.

In PC12 cells cultured in low-serum medium, the PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production of control group was decreased gradually from about 45% on day 0 to about 20% on day 6 (Fig. 3.11). For the NGF-treated group, the PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production

was more or less the same as the control group (Fig. 3.11). Therefore, NGF appeared not to influence the EP3 receptor response, and both the control and NGF-treated groups showed a similar decreasing trend of PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production, as a result, the apparent decrease of EP3 receptor response observed in section 3.1 might possibly be due to the reduced serum supply to PC12 cells after 32 h incubation.

3.2.2 Effect of NGF on basal and forskolin-stimulated [^{3}H]cAMP production

Although NGF appeared not to influence the EP3 receptor response over 6 days treatment, we noticed that NGF did affect the basal and forskolin-stimulated [^{3}H]cAMP production within this time course. In control PC12 cells cultured in complete culture medium, the basal [^{3}H]cAMP production remained low from day 0 to day 2, then it showed an increasing trend to day 6 (Fig. 3.12). Upon stimulation with forskolin, the [^{3}H]cAMP production was increased, however, there was no significant difference between the basal and forskolin-stimulated [^{3}H]cAMP production from day 0 to day 2. In contrast, the level of forskolin-stimulated [^{3}H]cAMP production was significantly higher than the basal from day 4 to day 6 (Fig. 3.12). For the NGF-treated group, the basal [^{3}H]cAMP production was identical to that of control group (Fig. 3.12). The forskolin-stimulated [^{3}H]cAMP production

of the NGF-treated group followed a similar trend as the control group, and was significantly higher than the basal on day 4 and day 6 (Fig. 3.12). Moreover, a striking effect was observed on day 4, showing that the NGF-treated group had a much greater forskolin-stimulated [^{3}H]cAMP production than the control group, and it remained higher on day 6, though the difference was no longer statistically significant (Fig. 3.12).

In control PC12 cells cultured in low-serum medium, the basal [^{3}H]cAMP production remained low from day 0 to day 6 (Fig. 3.13). Forskolin increased the [^{3}H]cAMP production in control group, however, there was no significant difference between the basal and forskolin-stimulated [^{3}H]cAMP production from day 0 to day 6 (Fig. 3.13). For the NGF-treated group, the basal [^{3}H]cAMP production was similar to the control group (Fig. 3.13). The forskolin-stimulated [^{3}H]cAMP production of the NGF-treated group was significant higher than the basal on day 4 and day 6 (Fig. 3.13). In addition, the forskolin-stimulated [^{3}H]cAMP production of the NGF-treated group was significantly greater than that of control group (Fig. 3.13).

PC12 cells behaved slightly differently in high-serum conditions compared with low-serum condition. For the control PC12 cells, the basal and forskolin-stimulated [^{3}H]cAMP production were similar in either high- or low-serum condition from day 0 to day 4, but these responses were significantly higher for cells maintained in

high-serum condition on day 6 (Fig. 3.14). Basal [^{3}H]cAMP production of NGF-treated PC12 cells was also significantly higher when cells were maintained in high-serum condition on day 6 (Fig. 3.15). Moreover, forskolin-stimulated [^{3}H]cAMP production of NGF-treated PC12 cells in high-serum condition was slightly higher than that in low-serum condition on day 4 and day 6 (Fig. 3.15). One of the possible explanations is gene transcription responsible for cAMP production, such as AC, is suppressed in low-serum condition. Therefore, PC12 cells tend to have a greater basal and forskolin-stimulated [^{3}H]cAMP production when maintained in high-serum condition.

The enhanced forskolin response in NGF-treated PC12 cells might due to the survival promoting effect of NGF. Therefore, the viability of PC12 cells maintained in low-serum medium on day 6 was determined by trypan blue exclusion test. There was no significant difference in the total number of cells between control and NGF-treated group, either before or after 30 min incubation with HBS or forskolin (Fig. 3.14A). Moreover, before the incubation with HBS or forskolin, the percentage of viable cells in both the control and NGF-treated group was over 90%, and forskolin did not influence the cell viability after 30 min incubation (Fig. 3.14B). Therefore, we can exclude the possibility that NGF enhanced forskolin response by promoting survival of PC12 cells.

To summarize the above results, it is obvious that NGF did not have any significant effect on the basal [^{3}H]cAMP production, but can dramatically augment the forskolin-stimulated [^{3}H]cAMP production of PC12 cells in either high or low serum conditions. Interestingly, as the enhanced forskolin response was only observed after a long NGF treatment, it might be due to the regulation of gene transcription and protein expression by NGF in PC12 cells (Gunning et al., 1981a). For example, NGF might promote the gene and protein expression of AC and lead to an enhanced forskolin-stimulated [^{3}H]cAMP production of PC12 cells, and the gene expression of AC will be discussed in section 3.2.5.

3.2.3 Acute effect of NGF on [^{3}H]cAMP production

In control PC12 cells cultured in low-serum medium on day 0, the 30 min incubation with NGF did not directly stimulate [^{3}H]cAMP production, and did not enhance forskolin-stimulated [^{3}H]cAMP production (Fig. 3.17A). Similar observations were obtained for control or NGF-treated PC12 cells on day 6 (Fig. 3.17B & C).

NGF alone did not stimulate AC directly through the activation of its receptor. Also, NGF did not augment the forskolin response acutely. These results further suggest that the enhanced forskolin-stimulated [^{3}H]cAMP production of NGF-treated

PC12 cells in section 3.2.2 was caused by the chronic effect of NGF on gene and protein expression.

3.2.4 Effect of NGF withdrawal on basal and forskolin-stimulated [^{3}H]cAMP production

We have demonstrated that a chronic treatment of PC12 cells with NGF could enhance forskolin-stimulated [^{3}H]cAMP production, and in this part of study, we aim to determine if a continued supply of NGF is necessary to maintain the enhanced forskolin response. In addition to a continued treatment with NGF over 6 days, NGF was removed on day 2 or day 4 to determine the effect of NGF withdrawal on the forskolin-stimulated [^{3}H]cAMP production in PC12 cells maintained in low-serum medium.

A consistent observation was obtained as before, the forskolin-stimulated [^{3}H]cAMP production of control and NGF-treated group increased from day 0 to day 6, with NGF-treated group having a significantly higher [^{3}H]cAMP production than control group on day 4 and day 6 (Fig. 3.18). When NGF was removed on day 2, there was no subsequent enhanced response of PC12 cells to forskolin on day 4, and the forskolin-stimulated [^{3}H]cAMP production ended up at a similar level as the control group on day 6 (Fig. 3.18). In contrast, the enhanced response to forskolin

was maintained on day 6 when NGF was removed on day 4 (Fig. 3.18).

We hypothesized that chronic NGF is required to promote gene expression responsible for the increase of forskolin response in PC12 cells, and it is a delayed response as the enhanced forskolin response was first observed after 4 days NGF treatment. Also, the initiation of expression of those genes are dependent on NGF but become independent of NGF at the later stage, as noted by the persistent enhanced forskolin response after 4 days NGF treatment but not after just 2 days NGF treatment.

3.2.5 Effect of NGF on adenylyl cyclase gene expression

We hypothesized that an increase of AC expression is one of the possible explanations to account for the enhanced forskolin-stimulated [^{3}H]cAMP production triggered by NGF in PC12 cells. As mentioned in section 1.8, AC is divided into Group I to Group IV, and Group IV AC is not sensitive to forskolin (Simonds, 1999). We have performed a preliminary study on how NGF affected the mRNA expression of AC2, AC6 and AC8 (representative members of Group II, I and III AC, respectively) in PC12 cells by RT-PCR.

We found that only AC6 mRNA was expressed in either control or NGF-treated PC12 cells as signal was detected at 495 bp (Fig. 3.19). In order to prove that the

absence of AC2 and AC8 expression in PC12 cells was not due to a problem with the primers, rat brain tissue was used as the positive control because they express AC2, AC6 and AC8 (Delmeire et al., 2003). Signals corresponding to all the three AC isoforms were detected in rat brain tissue, which confirmed that AC2 and AC8 were really absent in PC12 cells (Fig. 3.19).

To study the change of AC6 mRNA expression in PC12 cells, signals of AC6 expression was normalized against the signals of GAPDH. The AC6 mRNA expression remained stable from day 0 to day 6 (Fig. 3.20A). However, A6 mRNA expression was up-regulated after 2 days NGF treatment, but returned to control level on day 4 and then slightly decreased on day 6 (Fig. 3.20A). When NGF was removed on day 2, the degree of decrease of AC6 mRNA expression was smaller than the NGF-treated group on day 4, and ended up at a similar level as the control group (Fig. 3.20A). In contrast, the down-regulation of AC6 mRNA expression was maintained on day 6 when NGF was removed on day 4 (Fig. 3.20A). We also expressed the above data without normalized against the signals of GAPDH, and NGF did not up-regulate AC6 expression on day 2, but still down-regulate AC6 expression on day 4 and day 6 (Fig. 3.20B). However, as the intensity of signal depends on the exposure time, it is more appropriate to express the data with normalized against the signals of GAPDH.

These results appear to contradict to our hypothesis. However, this is only a preliminary study so it is better not to overweight on the data. Also, the other AC isoforms might be up-regulated instead of AC6 by NGF in PC12 cells to account for the enhanced forskolin-stimulated [^{3}H]cAMP production. We will therefore have to investigate the expression of all AC isoforms before coming to any definite conclusion.

3.2.6 Discussion

We found that NGF appeared not to change the degree of EP3 receptor response in PC12 cells maintained in high- or low-serum containing medium over 6 days treatment. However, we noticed that the forskolin-stimulated [^{3}H]cAMP production was increased in both control and NGF-treated PC12 cells cultured in high or low serum condition over 6 days treatment, and the NGF-treated group appeared to increase to a greater extent. As a result, it might be more difficult for PGE_2 to inhibit forskolin-stimulated [^{3}H]cAMP production at the later stage of this experiment. Since the forskolin-stimulated [^{3}H]cAMP production was different in the control and NGF-treated group, there is limitation using this experimental setup to determine the change of EP3 receptor response caused by NGF and we have to be conservative when interpreting these results. Perhaps, the radio-ligand receptor binding assay

using [^{3}H]PGE_2 is a better approach to study the effect of NGF on EP3 receptor expression in PC12 cells.

We also demonstrated that NGF did not induce [^{3}H]cAMP production directly through the activation of its receptors in PC12 cells, as short term treatment with NGF did not affect the basal or forskolin-stimulated [^{3}H]cAMP production. Moreover, there was no significant difference of forskolin-stimulated [^{3}H]cAMP production between control and NGF-treated cells from day 0 to day 2, but the response was significantly higher in NGF-treated cells from day 4 to day 6. Therefore, chronic NGF treatment enhanced forskolin-stimulated [^{3}H]cAMP production probably through the induction of AC expression, because the enhanced forskolin response was persistent after 4 days NGF treatment but not after 2 days NGF treatment. We speculated that the initiation of increase of AC expression in PC12 cells was dependent on NGF, however the maintenance of AC level became NGF-independent, provided that cells had received more than 2 days of NGF treatment.

Finally, we were able to detect AC6 in PC12 cells, but AC2 and AC8 were absent. Although the change of AC6 expression did not match with the time course of enhanced forskolin-stimulated [^{3}H]cAMP production, as AC6 expression was not up-regulated in NGF-treated PC12 cells on day 4 and day 6, the enhanced

forskolin-stimulated [^{3}H]cAMP production might be related to the change of other AC isoforms.

To summarize this part of study, we have demonstrated that chronic NGF treatment increased [^{3}H]cAMP production of PC12 cells possibly by increasing AC expression. The importance of elevating cAMP production in differentiation of PC12 cells will require further study.

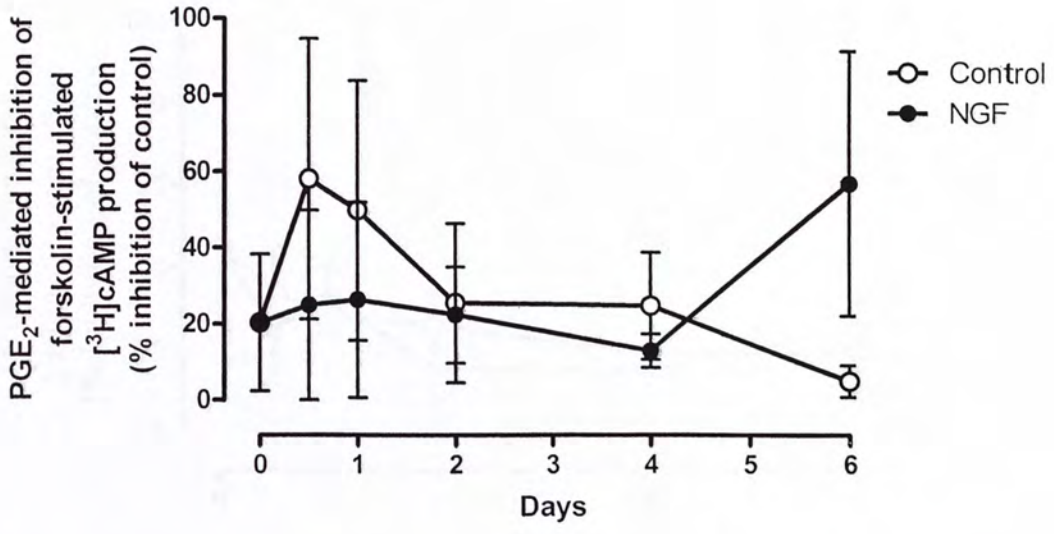


Fig. 3.10. The change of PGE_2-mediated inhibition of forskolin-stimulated $[^3H]$cAMP production by PC12 cells maintained in complete culture medium. PC12 cells were maintained in complete culture medium (± NGF, 50 ng/ml) over 6 days, and medium (± NGF) renewed every 2 days. After pre-labelling with $[^3H]$adenine, PC12 cells were incubated with HBS or PGE_2 (1 μM) in the presence or absence of forskolin (1 μM) for 30 min. The forskolin-stimulated $[^3H]$cAMP production in control or NGF-treated cells treated with HBS only was defined as 100%. Results represent mean ± S.E.M. of three independent experiments performed in duplicate.

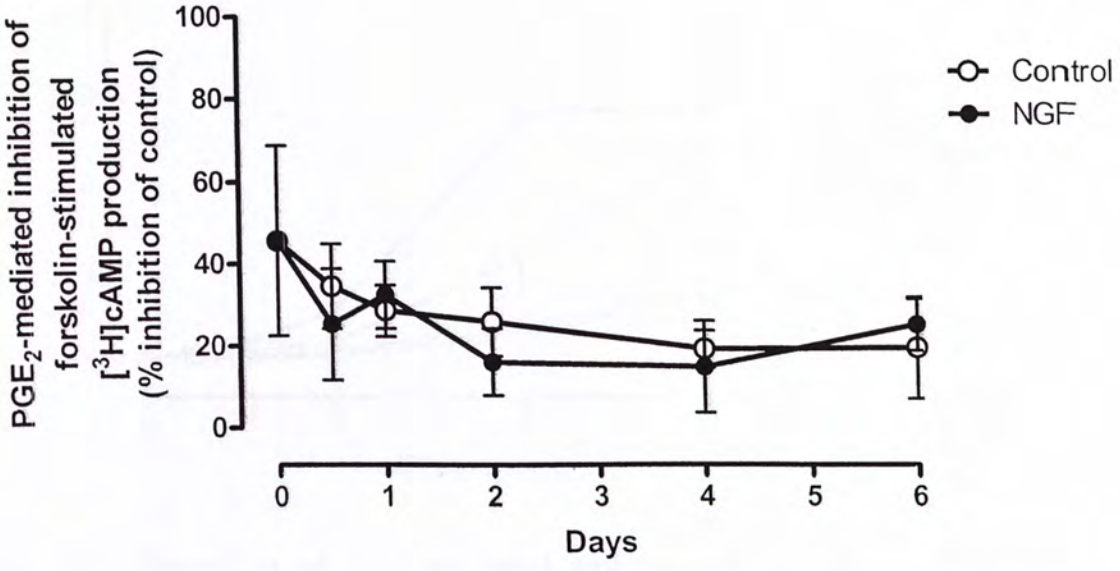


Fig. 3.11. The change of PGE_2-mediated inhibition of forskolin-stimulated $[^3H]$cAMP production by PC12 cells maintained in low-serum medium. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml) over 6 days, and medium (± NGF) renewed every 2 days. After pre-labelling with $[^3H]$adenine, PC12 cells were incubated with HBS or PGE_2 (1 μM) in the presence or absence of forskolin (1 μM) for 30 min. The forskolin-stimulated $[^3H]$cAMP production in control or NGF-treated cells treated with HBS only was defined as 100%. Results represent mean ± S.E.M. of three independent experiments performed in duplicate.

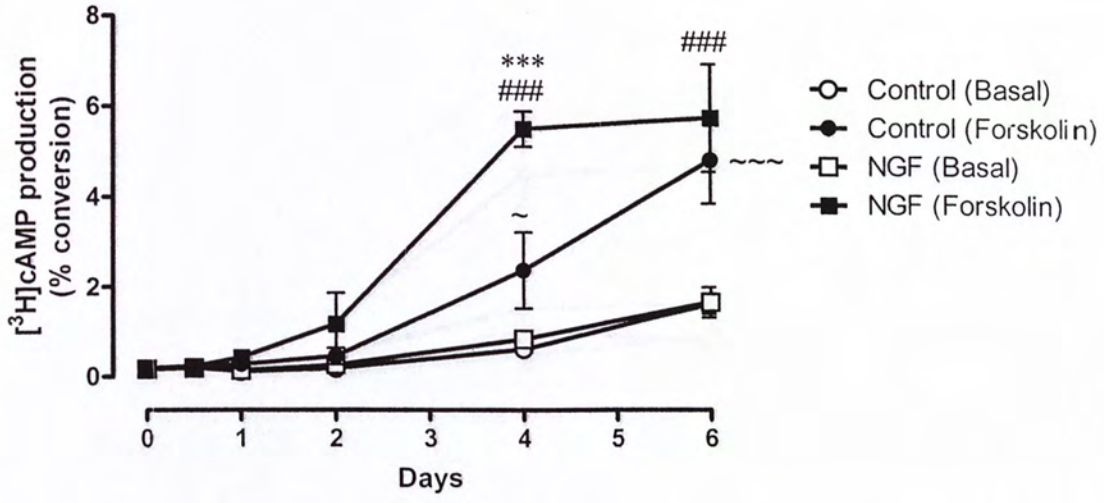


Fig. 3.12. The effect of NGF on basal and forskolin-stimulated [^{3}H]cAMP production by PC12 cells maintained in complete culture medium. Data obtained from Fig. 3.10. (Data analyzed with two-way ANOVA with Bonferroni post test. ***P<0.001, compared with forskolin-response of control group. ###P<0.001, compared with basal of NGF group. ~P<0.05, ~~~P<0.001, compared with basal of control group.)

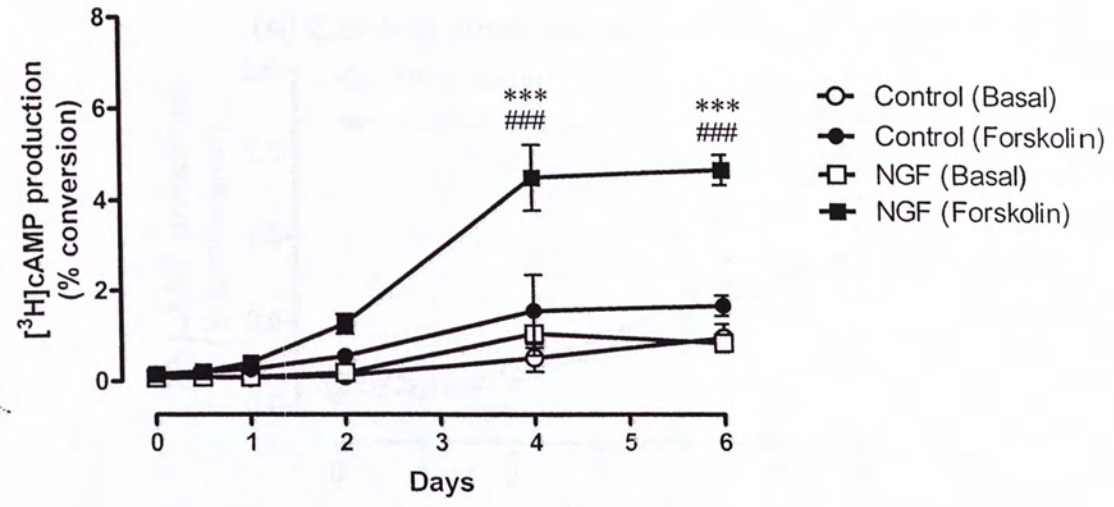


Fig. 3.13. The effect of NGF on basal and forskolin-stimulated [^{3}H]cAMP production of PC12 cells maintained in low-serum medium. Data obtained from Fig. 3.11. (Data analyzed with two-way ANOVA with Bonferroni post test. ***P<0.001, compared with forskolin-response of control group. ###P<0.001, compared with basal of NGF group.)

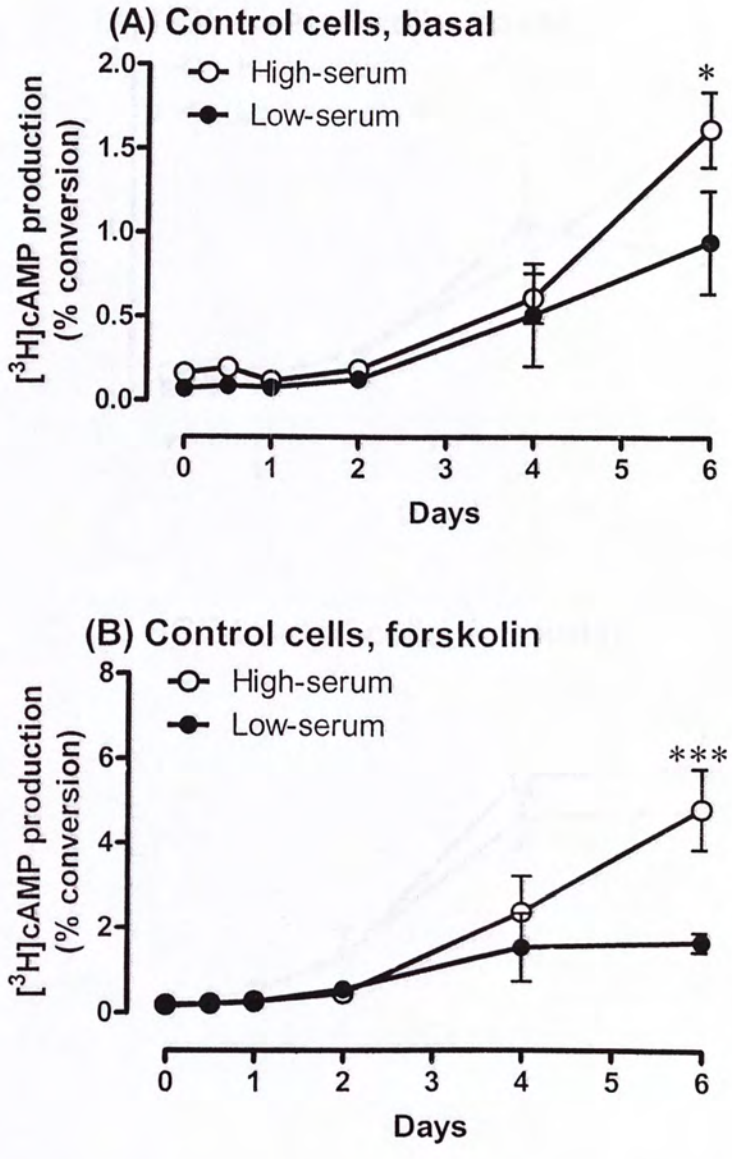


Fig. 3.14. Comparison of basal and forskolin-stimulated [^{3}H]cAMP production of control PC12 cells maintained in complete culture medium or low-serum medium. Data obtained from Fig. 3.10 and 3.11. (A) Basal [^{3}H]cAMP production. (B) Forskolin-stimulated [^{3}H]cAMP production. (***P<0.001, two-way ANOVA with Bonferroni post test.)

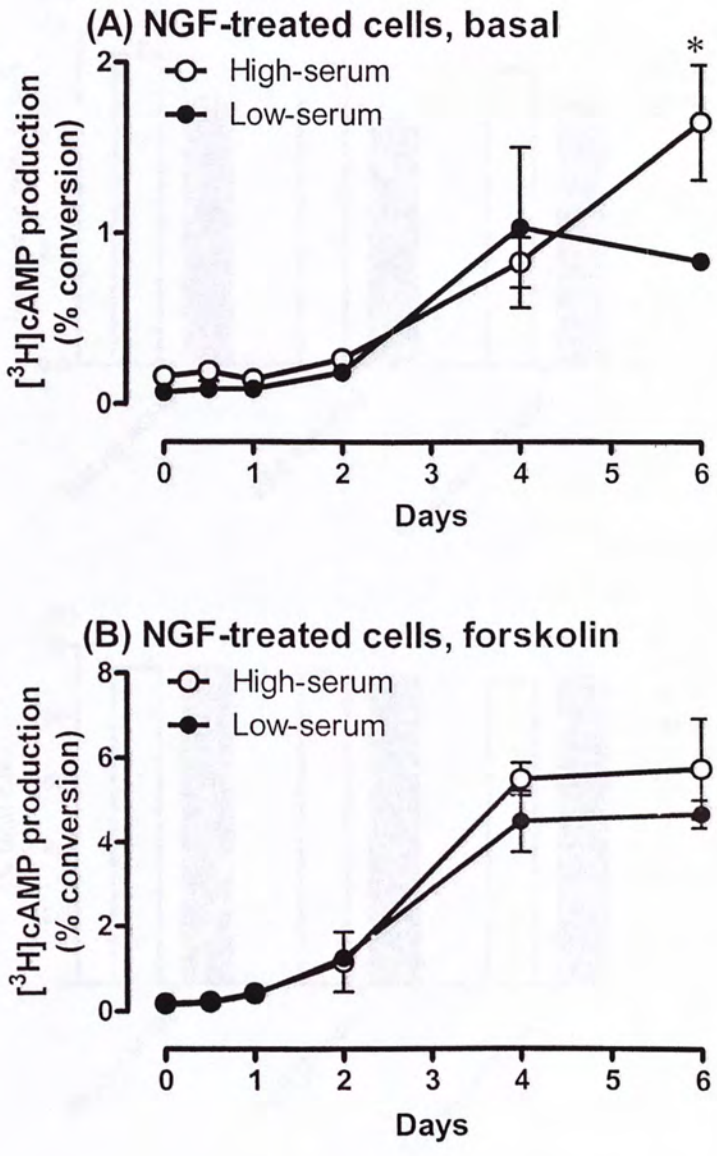


Fig. 3.15. Comparison of basal and forskolin-stimulated [^{3}H]cAMP production of NGF-treated PC12 cells maintained in complete culture medium or low-serum medium. Data obtained from Fig. 3.10 and 3.11. (A) Basal [^{3}H]cAMP production. (B) Forskolin-stimulated [^{3}H]cAMP production. (***P<0.001, two-way ANOVA with Bonferroni post test.)

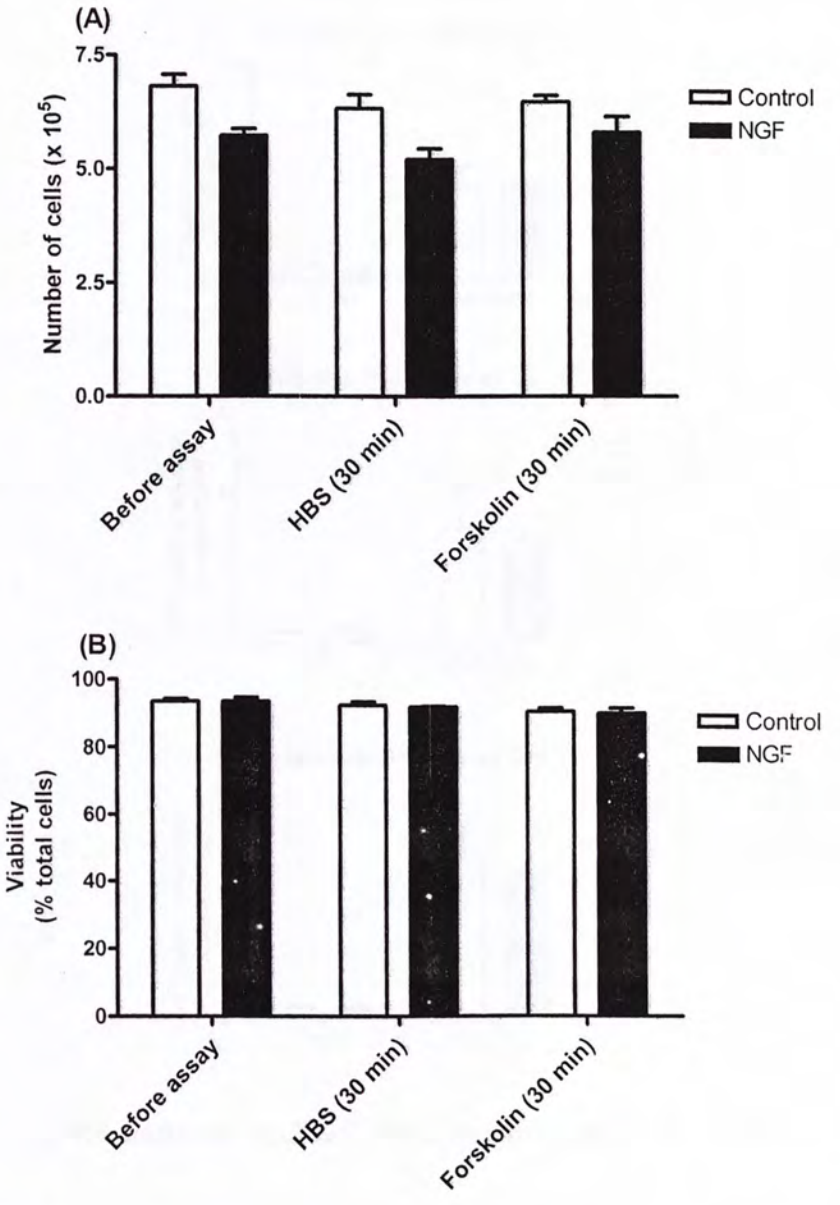


Fig. 3.16. Characteristics of PC12 cells on Day 6 as used in cAMP assay. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml) for 6 days, and medium (± NGF) renewed every 2 days. (A) Total cell number was counted with hemocytometer and (B) the cell viability was determined by trypan blue exclusion test, before and after incubation with HBS or forskolin (1 μM) for 30 min. Results represent mean ± S.E.M., from one experiment performed in triplicate.

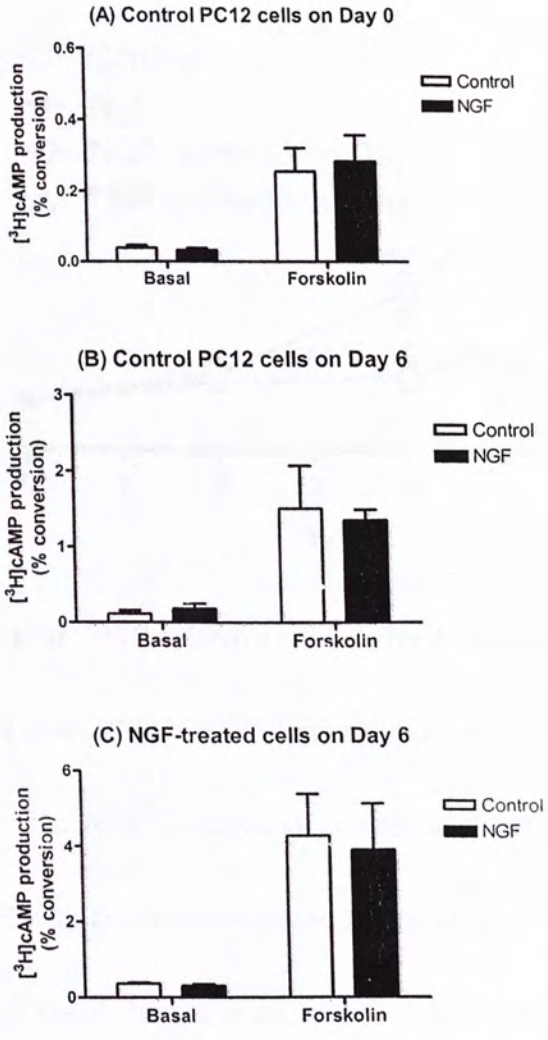


Fig. 3.17. Acute addition of NGF did not stimulate [^{3}H]cAMP production by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml), and medium renewed every 2 days. During the assay, cells were incubated with HBS or NGF (50 ng/ml) in the presence or absence of forskolin (1 μM) for 30 min. (A) Control cells on Day 0, (B) Control cells on Day 6, (C) NGF-treated cells on Day 6. Results represent mean ± S.E.M. of three independent experiments performed in triplicate.

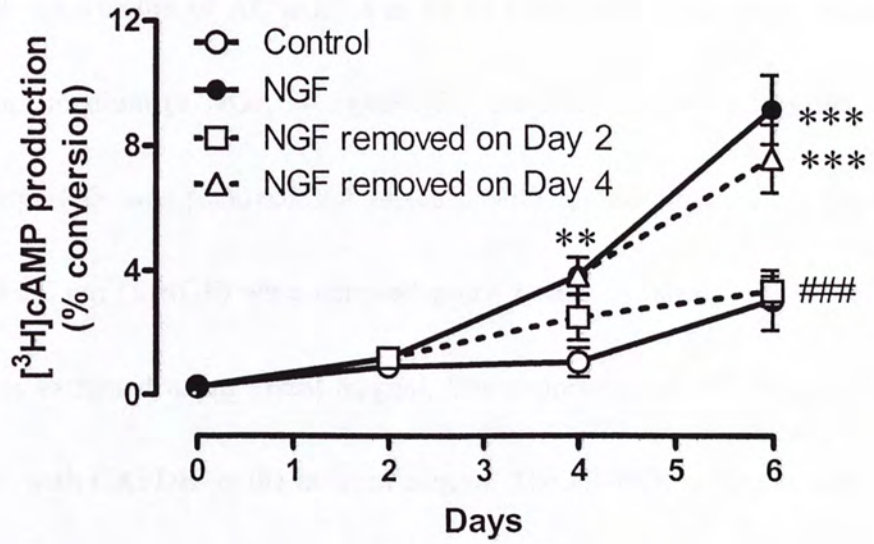
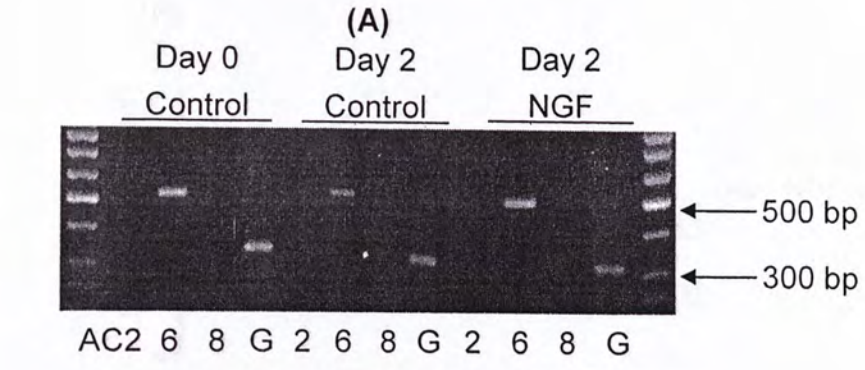
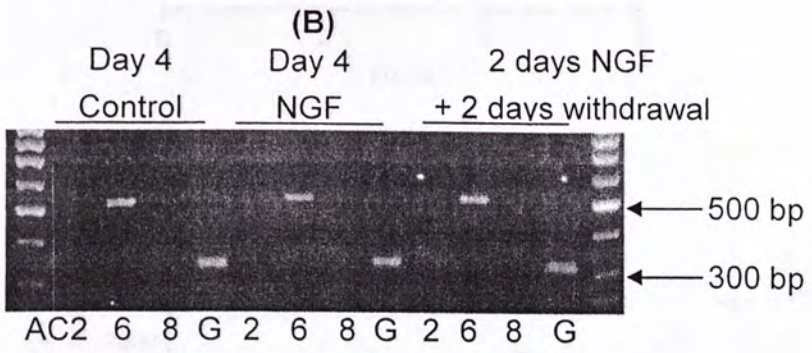
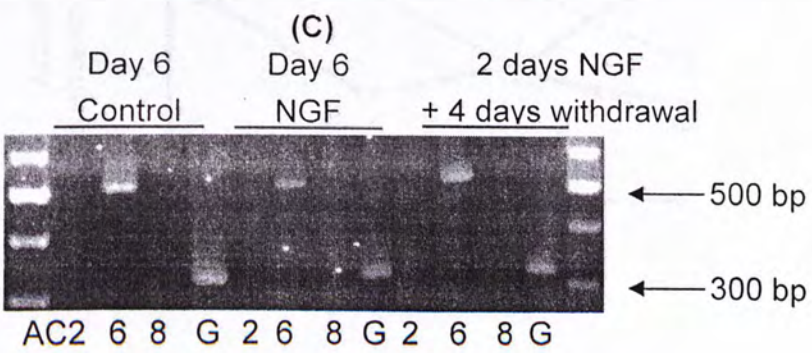


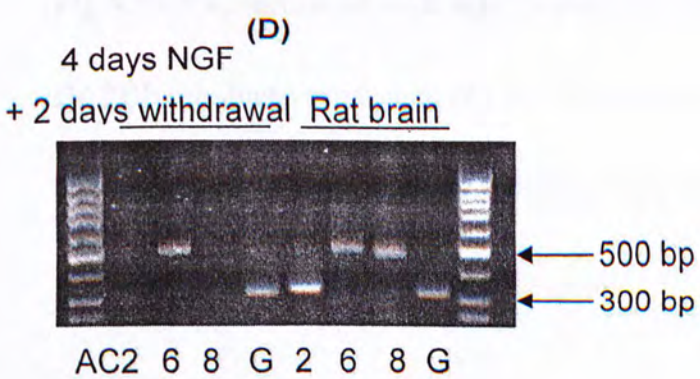
Fig. 3.18. The effect of NGF withdrawal on forskolin-stimulated [^{3}H]cAMP production by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). For the NGF withdrawal groups, medium containing NGF was removed and replaced with low-serum medium for the later period. Medium (± NGF) were renewed every 2 days. Results represent mean ± S.E.M. of three independent experiments performed in duplicate. (Data analyzed with two-way ANOVA with Bonferroni post test. $^{**}P<0.01$, $^{***}P<0.001$, compared with control group. ###$P<0.001$, compared with NGF group.)

Fig 3.19. Expression of AC mRNA in PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). For the NGF withdrawal groups, medium containing NGF was removed and replaced with low-serum medium for the later period. Medium (± NGF) were renewed every 2 days. At specific time points, total RNA was extracted using Trizol reagent. The expression of AC was performed by RT-PCR, with GAPDH as the internal control. The RT-PCR products were resolved by electrophoresis in 2.2% agarose gel and visualized by ethidium bromide staining and subsequent UV illumination. Rat brain tissue was used as positive control, and the expected RT-PCR products sizes of AC2, AC6, AC8 and GAPDH were 334, 495, 478 and 317 bp, respectively.


(A)
Day 0
Control
Day 2
Control
Day 2
NGF
500 bp
300 bp
AC2 6 8 G 2 6 8 G 2 6 8 G


(B)
Day 4
Control
Day 4
NGF
2 days NGF
+ 2 days withdrawal
500 bp
300 bp
AC2 6 8 G 2 6 8 G 2 6 8 G


(C)
Day 6
Control
Day 6
NGF
2 days NGF
+ 4 days withdrawal
500 bp
300 bp
AC2 6 8 G 2 6 8 G 2 6 8 G


(D)
4 days NGF
+ 2 days withdrawal
Rat brain
500 bp
300 bp
AC2 6 8 G 2 6 8 G

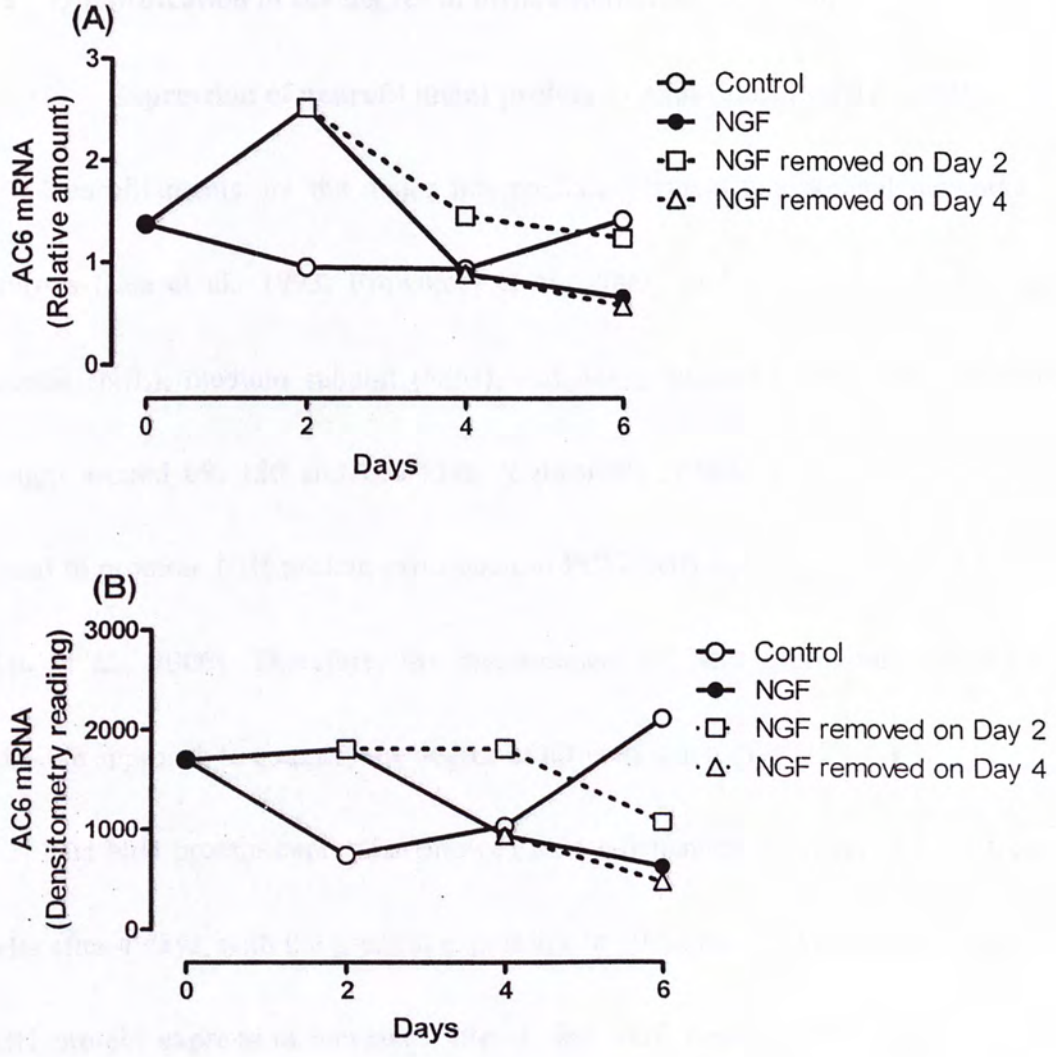


Fig 3.20. Expression of AC6 mRNA in PC12 cells. The band intensity of AC6 and GAPDH products were analyzed by Scion Image. The AC6 mRNA expression is expressed (A) relative to the level of GAPDH mRNA or (B) as the raw densitometry reading. Results come from one experiment.

3.3 Quantification of the degree of differentiation of PC12 cells

3.3.1 Expression of neurofilament protein as a marker of differentiation

Neurofilaments are the major intermediate filament cytoskeletal networks of neurons (Lee et al., 1993; Brownlees et al., 2000), and are composed of a light subunit (NfL), medium subunit (NfM), and heavy subunit (NfH), with molecular weight around 68, 150 and 200 kDa, respectively (Elder et al., 1998). NGF was found to promote NfH protein expression in PC12 cells in a time-dependent manner (Liu et al., 2006). Therefore, the measurement of NfH expression would be a possible approach to quantify the degree of differentiation of PC12 cells.

The NfH protein expression showed a dose-dependent increase in NGF-treated cells after 4 days, with the greatest expression at 50 ng/ml of NGF (Fig. 3.21A). The NfH protein expression increased after 1 day NGF treatment (50 ng/ml), but the expression was not increased further afterward (Fig. 3.21B). When PC12 cells were treated with various concentrations of NGF for longer periods, we could not observe an obvious trend showing that the NfH protein expression was correlated to NGF exposure (Fig. 3.22). As a result, it is difficult to employ the measurement of NfH expression to quantify the degree of differentiation of PC12 cells in our system.

3.3.2 Neurite assays

Since the use of neurofilament protein expression as the marker of neuronal cell differentiation was not so successful, we adopted another method to quantify differentiation. A typical method to assess differentiation is by counting the number of cells extending neurites of lengths more than one cell diameter or by direct measurement of neurite length. We have performed this assay using two approaches, by manual assessment of PC12 cells under the observation of microscope and quantification of the captured cell images. In order to obtain clear images of PC12 cells without overcrowding, cells were plated at low density (5×10^4 cells/well) in 6-well plates.

3.3.2.1 Manual assessment of PC12 cells

The proportion of PC12 cells expressing neurites of length more than one cell diameter was determined over 14 days NGF treatment. As expected, the proportion of PC12 cells expressing neurites of the control group over the treatment period remained low at not more than 3% (Fig. 3.23). In contrast, the proportion of cells expressing neurites in the NGF-treated group increased in a time-dependent manner (Fig. 3.23). The results confirmed that NGF can promote differentiation of PC12 cells as expected (Perry et al., 2002; Attiah et al., 2003; Cappelletti et al., 2007).

However, there are at least two limitations of this assay. First, the manual assessment might be too subjective and is time-consuming (take about 20 min for each plate), cells have to be exposed to an environment which is unfavorable for their growth, including the drop of temperature and carbon dioxide content. Second, we cannot measure multiple parameters at the same time. We can only count the number of neurite-expressing cells at each time point, but cannot quantify the length of neurites, so we cannot get a comprehensive representation of the differentiation of PC12 cells.

3.3.2.2 Quantification of images of PC12 cells

Due to the limitation of the manual assessment method, we employed another method to quantify differentiation of PC12 cells. With the aid of a digital image capturing system, random fields of cells can be captured and analyzed later, which helps to minimize the exposure time of the cells in unfavorable environment. Also, it is possible to measure multiple parameters of the same group of cells. The captured cell images of control and NGF-treated PC12 cells were shown in Fig. 3.24.

We have studied the effect of NGF and its withdrawal on the neurite expression of PC12 cells. In our experiment, the control group did not appear to express neurites, while the NGF-treated group showed an increase in proportion of cells expressing neurites in a time-dependent manner, and about 80% of cells expressed neurites after

6 days treatment (Fig. 3.25), which implied that this method was more sensitive than the manual assessment of PC12 cells as only about 30% of cells expressed neurites after 6 days treatment using the latter method (Fig. 3.23). When NGF was removed on either day 2 or day 4, both groups showed a decrease in proportion of cells expressing neurites (Fig. 3.25). The average length of the longest neurite was also quantified. NGF significantly increased the neurite length after 2 days, and the neurite length increased in a time-dependent manner afterwards (Fig. 3.26). The neurite length decreased when NGF was removed on either day 2 or day 4 (Fig. 3.26). A similar pattern was observed when measuring the number of neurites expressed by PC12 cells (Fig. 3.27). The quantifications of the above three parameters were consistent with the reported founding showed that neurite stability of PC12 cells was decreased after removal of NGF (Attiah et al., 2003). In summary, we have demonstrated that NGF can promote differentiation of PC12 cells revealed by the increased proportion of neurite-expressing cells, increased length of neurite, and the increased average number of neurite expression. Also, the continual presence of NGF is required to maintain the existence of neurites in PC12 cells.

Several studies have reported that in PC12 cells stably expressing EP3B receptor isoform isolated from bovine adrenal chromaffin cells, treatment with EP3 receptor agonist of the NGF-treated cells can induce neurite retraction through a

PTX-insensitive pathway (Katoh et al., 1996; Katoh et al., 1998a; Aoki et al., 1999). Therefore, we hypothesized that PGE_2 might attenuate NGF-induced neurite outgrowth of PC12 cells. Conversely, blocking EP3 receptors with ONO-AE3-240 might enhance the NGF-induced neurite outgrowth of PC12 cells. The results suggested that treatment with PGE_2 alone, ONO-AE3-240 alone, or combination of these two compounds did not affect the proportion of neurite-expressing cells (Fig. 3.28) or the average length of longest neurite (Fig. 3.29). Neither treatment with PGE_2 alone, nor ONO-AE3-240 alone affected the average number of neurite expression of PC12 cells, however, it was not expected that the co-treatment of these two compounds could increase the average number of neurite expression (Fig. 3.30).

3.3.3 Discussion

In this part of study, we have established a proper method to quantify the differentiation of PC12 cells. The measurement of NfH protein expression is perhaps a more objective and less labor-intensive method than the neurite assay, however, it is only semi-quantitative, and due to the minimal induction of NfH protein expression by NGF in PC12 cells which limited the sensitivity of this assay, we failed to adopt the measurement of NfH protein expression as the marker of differentiation.

The manual assessment of PC12 cells appeared to be less sensitive than quantification of cell images, as the proportion of cells classified as neurite-expressing in the later method was much greater than the former method. The reason might be due to the estimation of cell diameter and neurite length by manual observation. Therefore, the degree of differentiation determined by quantification of cells images of PC12 cell was considered as the best method in our study.

The results suggest that NGF can promote differentiation of PC12 cells by increasing the proportion of neurite-expressing cells, length of neurite, and the average number of neurite expression, and these three parameters showed a decreasing trend no matter when NGF was removed. In contrast, in section 3.2.4, we found that the enhanced forskolin-stimulated [^{3}H]cAMP production was maintained when NGF was removed on day 4 but not on day 2, suggesting that cAMP was not related to the maintenance of the stability of neurites. Therefore, NGF is indispensable for the maintenance of neurites of PC12 cells through a cAMP-independent manner.

We have showed that NGF did not regulate the EP3 receptor expression in PC12 cells in section 3.2.1, we also demonstrated that neither activations of EP3 receptors using PGE_2 nor blocking EP3 receptors using ONO-AE3-240 could influence NGF-induced neurite outgrowth. This finding suggested that EP3 receptors are not

directly participating in the regulation of neurite outgrowth of NGF-treated PC12 cells. However, EP3 receptors might play other functional roles in PC12 cells, for example, the dopamine release was inhibited in PC12 cells by the activation of EP3 receptors (Nakamura et al., 1998). In summary, we have employed the neurite assay to monitor the degree of differentiation of PC12 cells and proved that the continual presence of NGF is required to maintain the stability of neurites in PC12 cells. And this approach can also be used to study the effect of other compounds which may influence NGF-induced differentiation of PC12 cells, as we found that neither PGE_2 nor ONO-AE3-240 could change the degree of NGF-induced differentiation of PC12 cells.

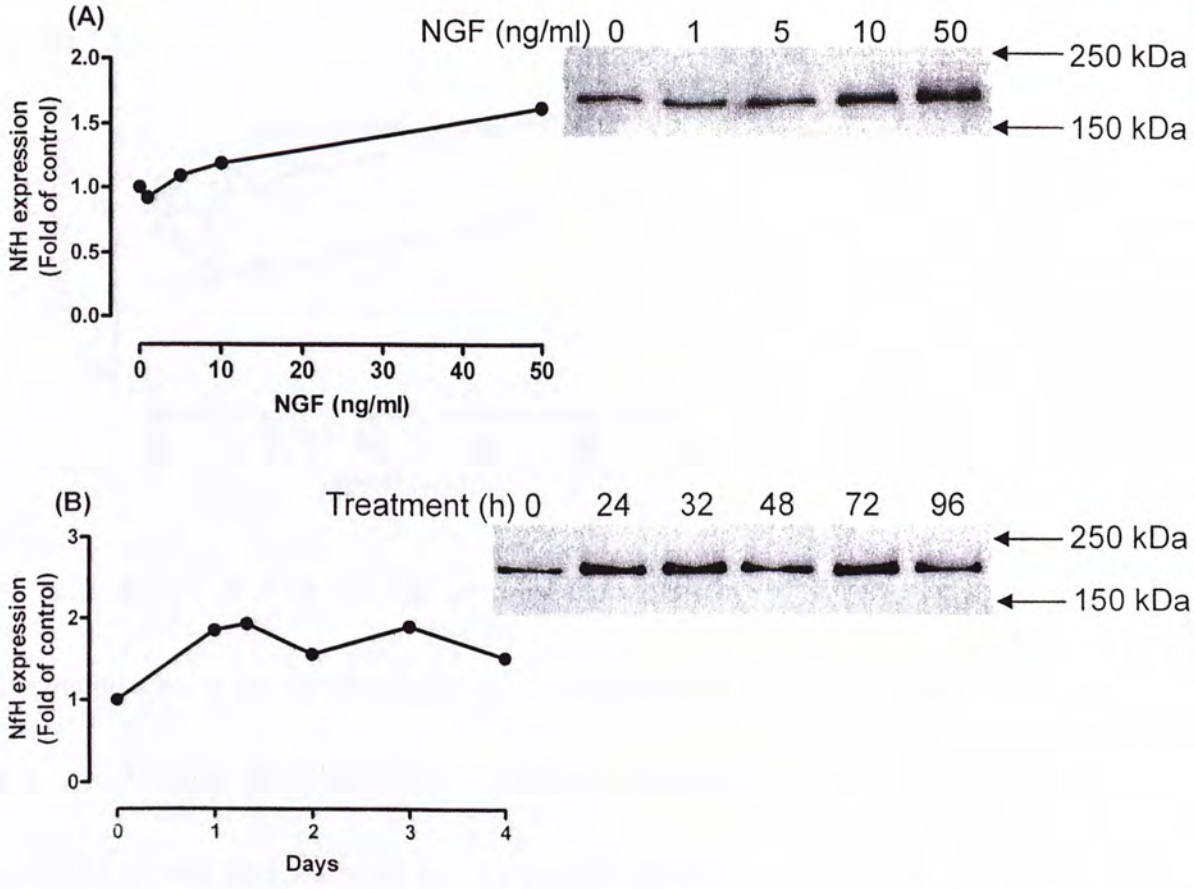


Fig. 3.21. Expression of neurofilament heavy chain (NfH) protein in PC12 cells. PC12 cells were maintained in low-serum medium and treated with (A) 0, 1, 5, 10 or 50 ng/ml NGF for 4 days or (B) 50 ng/ml NGF for 0 to 4 days. NGF and medium were renewed every 2 days. At the specific time, total protein was extracted and the protein concentration was determined by protein assay. Equal amount of protein (20 μg) of each sample were loaded onto 6% SDS-PAGE gel. The band intensities were determined by Scion Image. The NfH level was expressed as fold of increase of control (samples without NGF in (A) and at time zero in (B) were defined as control). Results come from one experiment.

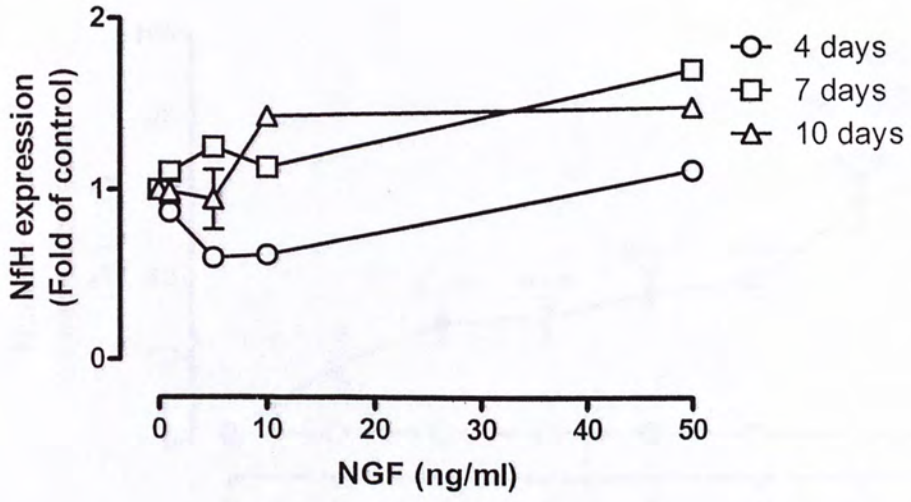


Fig. 3.22. Effect of NGF on NfH protein expression in PC12 cells. PC12 cells were maintained in low-serum medium and treated with 0, 1, 5, 10 or 50 ng/ml NGF for 4, 7 or 10 days. NGF and medium were renewed every 2 days. At the specific time, total protein was extracted and the protein concentration was determined by protein assay. Equal amount of protein (20 μg) of each sample were loaded onto 6% SDS-PAGE gel. The band intensities were determined by Scion Image. The NfH level was expressed as fold of increase of control (samples without NGF were defined as control). Results come from one experiment.

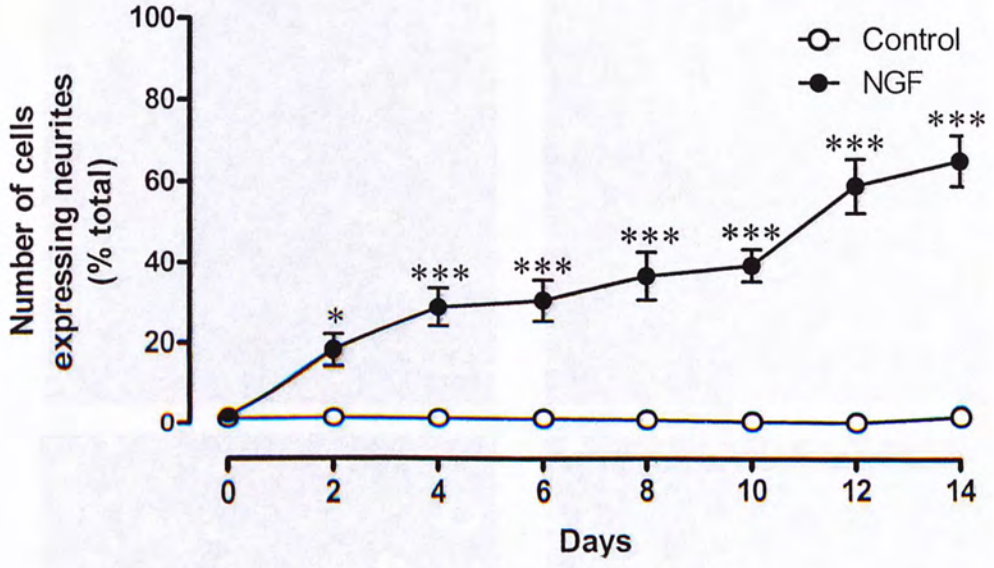


Fig. 3.23. Manual assessment of neurites expression by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml), and medium was renewed every 2 days. At specific time points, 100 cells per well were quantified under microscopic observation. Cells were considered as expressing neurites with neurite greater than one cell body in length. Results represent mean ± S.E.M. of three independent experiments performed in triplicate. (Data analyzed with two-way ANOVA with Bonferroni post test. *P<0.05, ***P<0.001, compared with control group.)

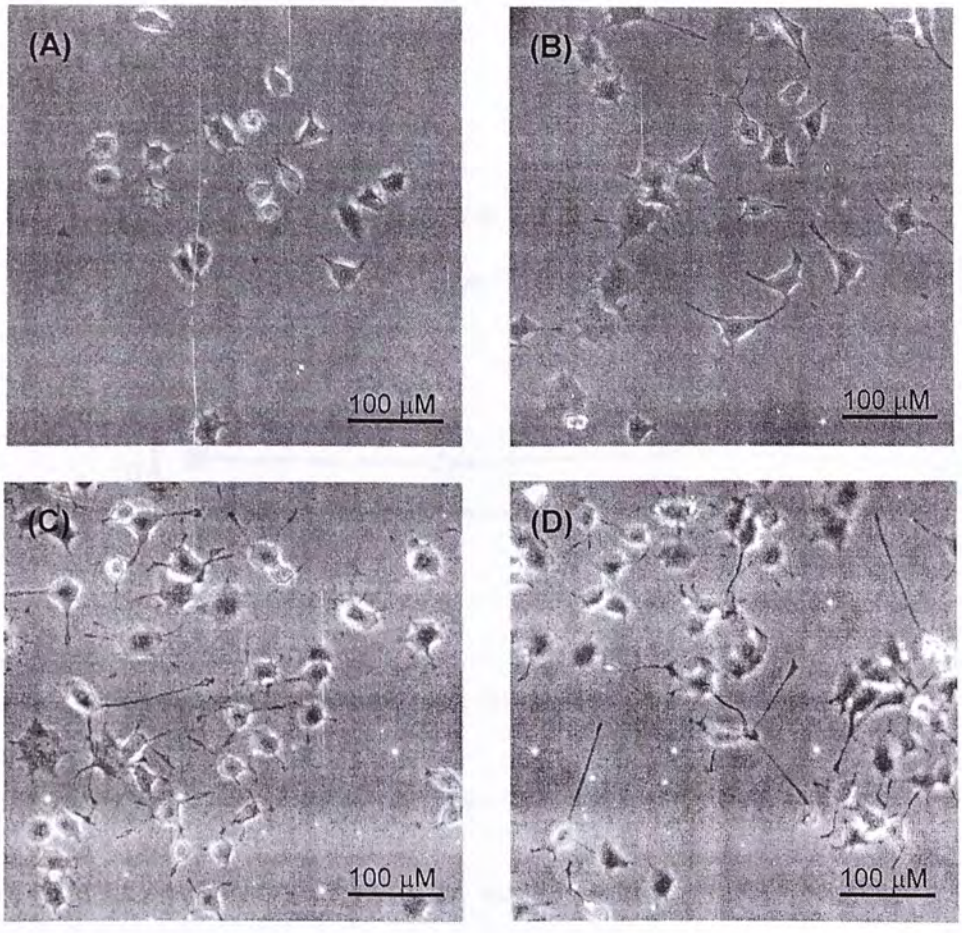


Fig. 3.24. Images of PC12 cells captured by digital camera. Images presented were corresponding to Fig. 3.25. (A) Control cells on Day 0, (B) NGF-treated cells on day 2, (C) on day 4, and (D) on day 6.

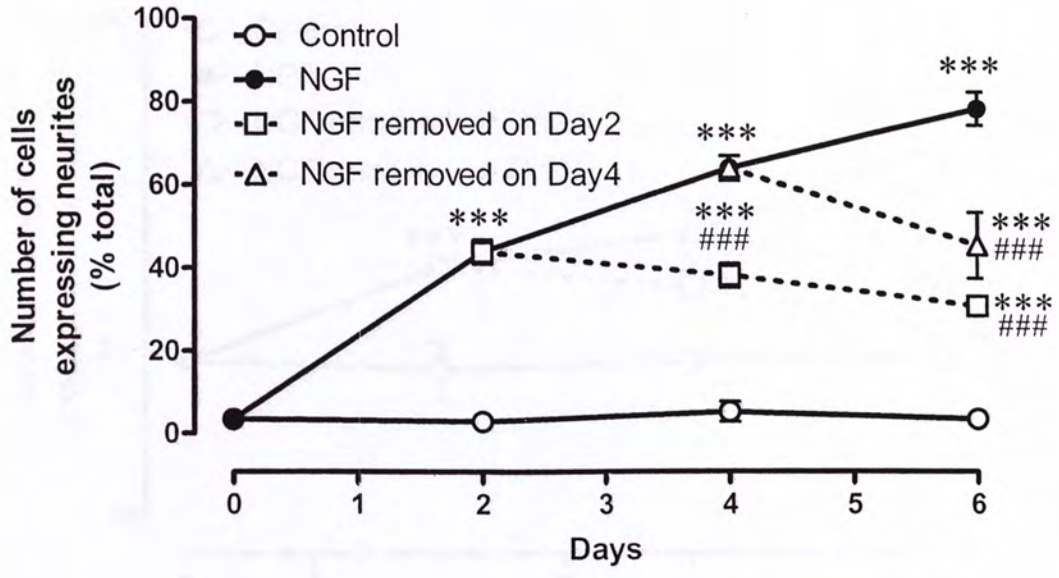


Fig. 3.25. The effect of NGF and its withdrawal on the number of PC12 cells expressing neurites. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). For the NGF withdrawal groups, medium containing NGF was removed and replaced with low-serum medium for the later period. Medium (± NGF) were renewed every 2 days. At specific time points, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Results represent mean ± S.E.M., from six independent wells and two independent experiments. (Data analyzed with two-way ANOVA with Bonferroni post test. ***P<0.001, compared with control group. ###P<0.001, compared with NGF group.)

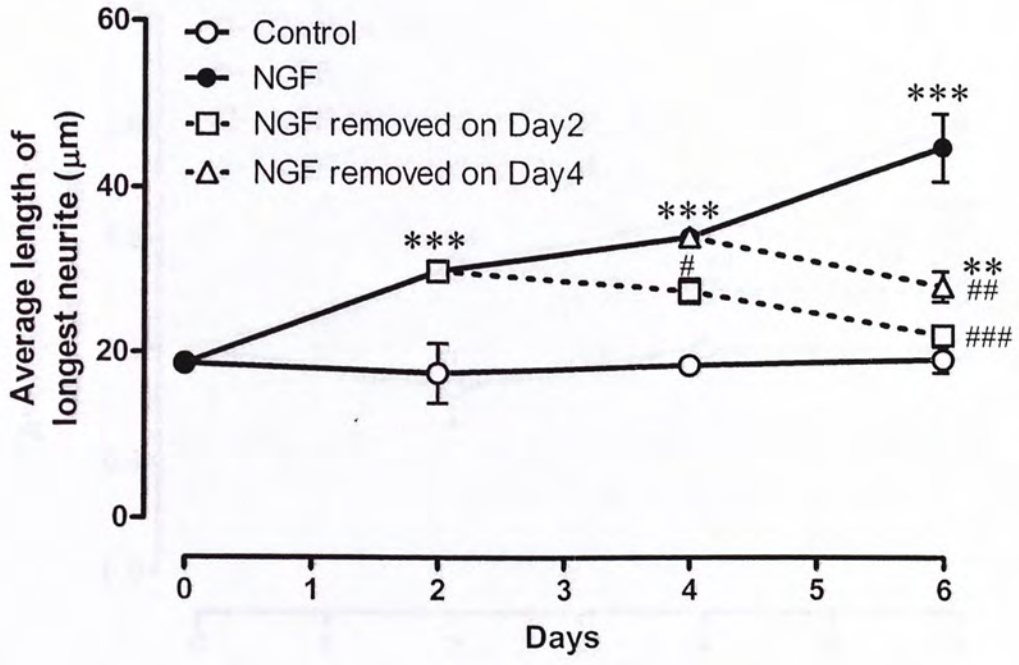


Fig. 3.26. The effect of NGF and its withdrawal on the length of neurites of PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). For the NGF withdrawal groups, medium containing NGF was removed and replaced with low-serum medium for the later period. Medium (± NGF) were renewed every 2 days. At specific time points, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Lengths of longest neurite of neurite-possessing cells were measured. Results represent mean ± S.E.M., from six independent wells and two independent experiments. (Data analyzed with two-way ANOVA with Bonferroni post test. $^{**}P<0.001$, $^{***}P<0.001$, compared with control group. $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$, compared with NGF group.)

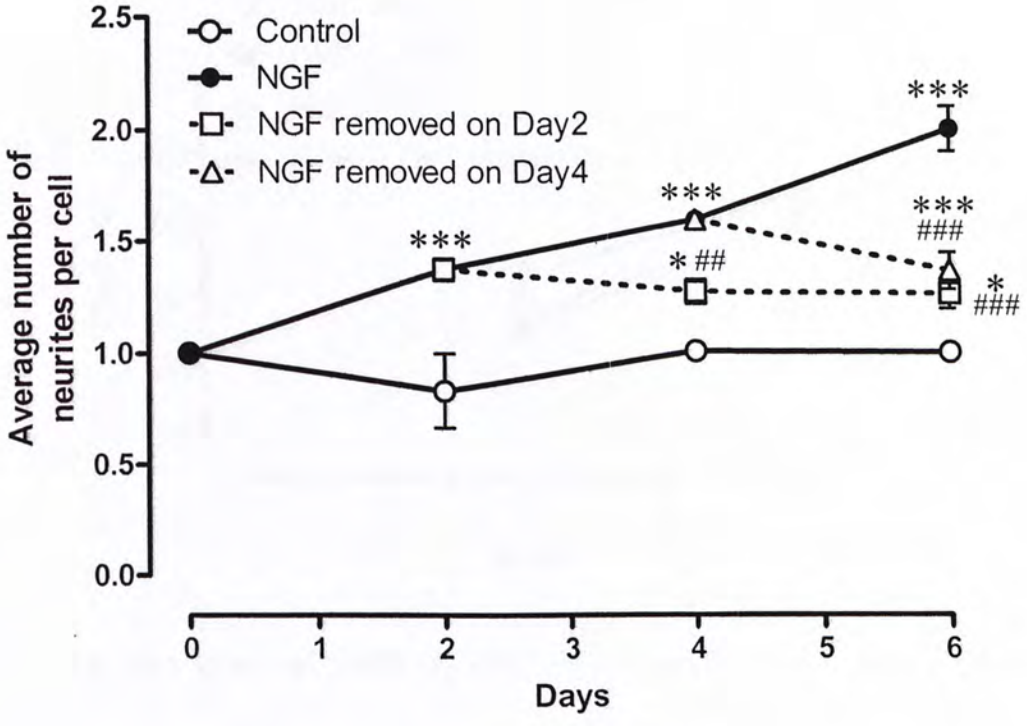


Fig. 3.27. The effect of NGF and its withdrawal on the number of neurites expressed by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). For the NGF withdrawal groups, medium containing NGF was removed and replaced with low-serum medium for the later period. Medium (± NGF) were renewed every 2 days. At specific time points, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Results represent mean ± S.E.M., from six independent wells and two independent experiments. (Data analyzed with two-way ANOVA with Bonferroni post test. *P<0.05, ***P<0.001, compared with control group. ##P<0.01, ###P<0.001, compared with NGF group.)

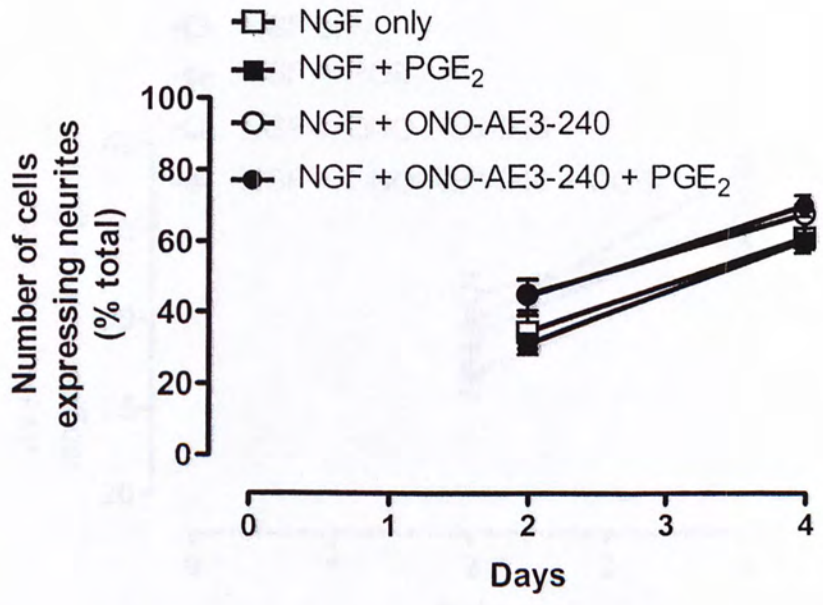


Fig. 3.28. The effect of ONO-AE3-240 on number of PC12 cells expressing neurites. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). ONO-AE3-240 (10^{-5} M) was added 3 h before the addition of NGF on Day 0. Drugs and medium were renewed every day. At specific time points, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Results represent mean ± S.E.M., from six independent wells and two independent experiments.

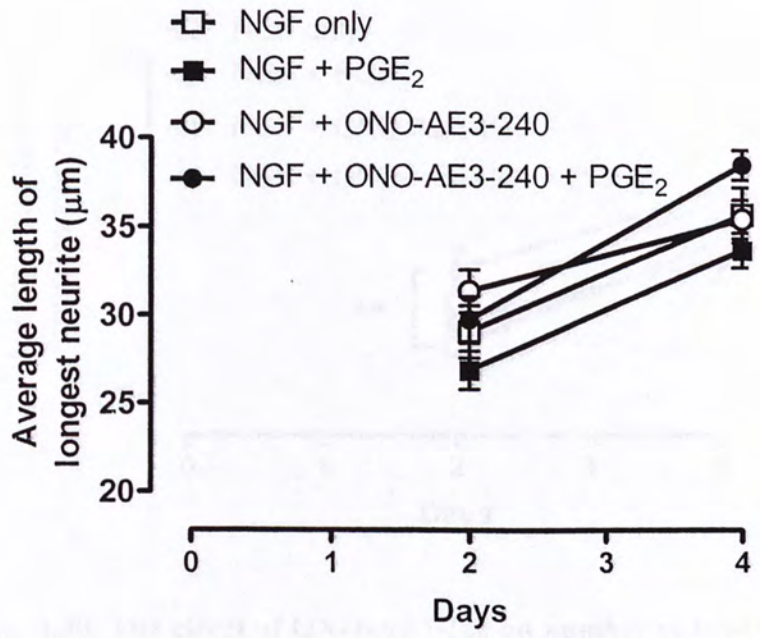


Fig. 3.29. The effect of ONO-AE3-240 on the length of neurites of PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). ONO-AE3-240 (10^{-5} M) was added 3 h before the addition of NGF on Day 0. Drugs and medium were renewed every day. At specific time, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Lengths of longest neurite of neurite-possessing cells were measured. Results represent mean ± S.E.M., from six independent wells and two independent experiments.

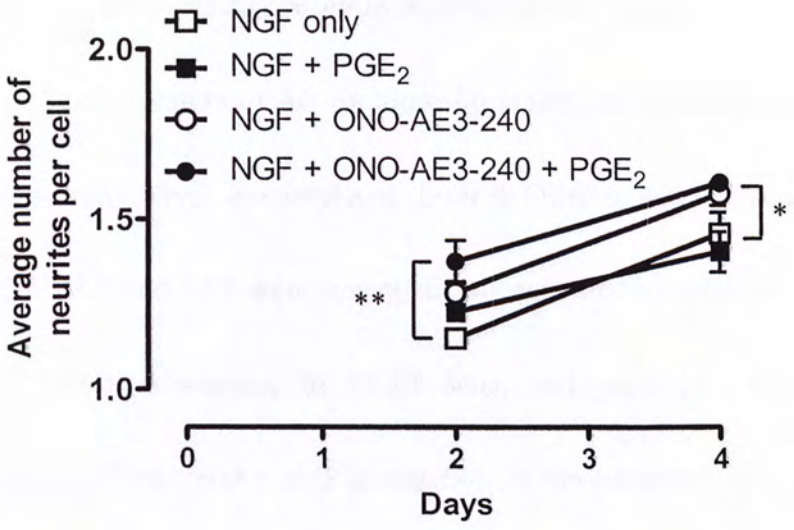


Fig. 3.30. The effect of ONO-AE3-240 on number of neurites expressed by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml). ONO-AE3-240 (10^{-5} M) was added 3 h before the addition of NGF on Day 0. Drugs and medium were renewed every day. At specific time, images were captured and 100 cells were quantified for each well. Cells were considered as expressing neurite with neurite greater than one cell body in length. Results represent mean ± S.E.M., from six independent wells and two independent experiments. (*P<0.05, **P<0.01, two-way ANOVA with Bonferroni post test.)

3.4 Adenosine A_{2A} receptor activity in PC12 cells

The activation of AC by forskolin is influenced by G_s proteins, which enhance AC activity when co-stimulated (Insel & Ostrom, 2003). For example, in Sf9 cells, AC2, AC5 and AC6 were synergistically activated by forskolin and G_s (Sutkowski et al., 1994). Moreover, in PC12 cells, endogenously produced adenosine also influenced the basal cAMP production as the treatment with adenosine deaminase (ADA), which metabolizes adenosine to inosine, or the presence of an A_{2A} receptor antagonist, were found to decrease the basal cAMP production (Roskoski & Roskoski, 1989).

In this part of the study, we aimed to explain the relationship between NGF and the enhanced forskolin-stimulated cAMP production by studying the involvement of A_{2A} receptors in the regulation of AC activity in our PC12 cells. Some reports suggested that NGF could down-regulate A_{2A} receptor expression in PC12 cells (Arslan et al., 1997; Nie et al., 1999). Therefore, we studied the effect of NGF on the A_{2A} receptor response over 6 days, and also investigated the effect of blocking A_{2A} receptor activation on forskolin-stimulated responses in PC12 cells.

3.4.1 Effect of NGF on A_{2A} receptor-mediated [^{3}H]cAMP production

The CGS21680-induced [^{3}H]cAMP production of the control and NGF-treated

group were similar, having a gradual increase of response, except that there was an apparent decrease in the response of the control group on day 4 (Fig. 3.31). However, this finding appeared to contradict several studies showing that NGF could induce the down-regulation of A_{2A} receptors, as revealed by the decrease of agonist-induced cAMP production and reduced amount of A_{2A} receptor mRNA and protein expression (Arslan et al., 1997; Nie et al., 1999). As NGF appeared not to regulate A_{2A} receptors response in our PC12 cells, the removal of NGF also did not affect the A_{2A} receptor response (Fig. 3.31). In contrast, for PC12 cells with A_{2A} receptor expression being down-regulated by NGF, the receptor expression was almost recovered completely after 2 days of NGF withdrawal (Nie et al., 1999).

To confirm that CGS21680 is really acting on A_{2A} receptor in PC12 cells, we have performed an antagonist study using the selective A_{2A} receptor antagonist, ZM241385 (Uustare et al., 2005). As expected, CGS21680 greatly increased the [^{3}H]cAMP production in untreated PC12 cells (Fig. 3.32). ZM241385 did not affect the basal [^{3}H]cAMP production, but strongly inhibited [^{3}H]cAMP production induced by CGS21680 (Fig. 3.32). In summary, our results suggest that A_{2A} receptors were expressed and NGF did not regulate A_{2A} receptor-mediated [^{3}H]cAMP production in our PC12 cells.

3.4.2 Synergistic activation of adenylyl cyclase by A_{2A} receptor and forskolin

It has been demonstrated that the activation of AC by forskolin in undifferentiated PC12 cells was greatly dependent on the presence of activated A_{2A} receptors, as A_{2A} receptor antagonists inhibited forskolin-stimulated cAMP production in a dose-dependent manner (Florio et al., 1999a). Conversely, adenosine which was applied exogenously, could facilitate the forskolin-stimulated cAMP production in PC12 cells (Florio et al., 1999a), which further highlighted the importance of the A_{2A} receptor on the forskolin effect on AC. In our experiment, we aimed to investigate if NGF has any effect on the association of A_{2A} receptor and forskolin on the activation of AC.

In undifferentiated PC12 cells, CGS21680 and forskolin were able to give about 4- and 6-fold increase of [^{3}H]cAMP production compared to the basal production, respectively (Fig. 3.33A). When the cells were treated with CGS21680 and forskolin simultaneously, [^{3}H]cAMP production was enhanced in a synergistic manner, giving about 51-fold increase when compared to the basal production (Fig. 3.33A). A similar pattern was observed in NGF-treated cells, and the co-treatment with CGS21680 and forskolin increased [^{3}H]cAMP production to a similar extent as in undifferentiated PC12 cells (Fig. 3.33B).

The forskolin-induced [^{3}H]cAMP production was also found to be sensitive to

ADA in PC12 cells (Florio et al., 1999b). Therefore, we have performed a preliminary experiment to test if the forskolin response was also sensitive to ADA in our PC12 cells. In untreated PC12 cells, ADA neither lowered the basal nor forskolin-stimulated [^{3}H]cAMP production (Fig. 3.34). As a result, our PC12 cells might not produce adenosine endogenously, or the endogenously produced adenosine might not play a significant role on the basal and forskolin-stimulated [^{3}H]cAMP production in our PC12 cells.

3.4.3 Chronic and acute effect of ADA and ZM241385 on [^{3}H]cAMP production

Since we have demonstrated that the exogenously applied A_{2A} receptor agonist could enhance the activation of AC by forskolin in our PC12 cells, and the forskolin response appeared not to be related to the endogenous production of adenosine as observed in the preliminary test in Fig. 3.34, we would like to further our study on how chronic or acute treatment with ADA or ZM241385 might affect the forskolin-stimulated [^{3}H]cAMP production in untreated or NGF-treated PC12 cells. The CGS21680 response was also measured in order to monitor the change of A_{2A} receptor response which might be correlated with the forskolin response.

3.4.3.1 Chronic effect of ADA and ZM241385

We study the chronic effect of ADA and ZM241385 in untreated or NGF-treated PC12 cells on day 4, because the enhanced forskolin-stimulated [^{3}H]cAMP production was first observed after 4 days NGF treatment. In untreated PC12 cells incubated with ADA or ZM241385 for 4 days, neither ADA nor ZM241385 affected the basal [^{3}H]cAMP production (Fig. 3.35A). Due to the variation of data, none of the forskolin or CGS21680 responses were classified as significant in control, ADA-treated or ZM241385-treated groups (Fig. 3.35A). Although ADA apparently enhanced the forskolin and CGS21680 response in untreated PC12 cells, but these responses were not significantly different from those in control or ZM241385-treated group (Fig. 3.35A).

In NGF-treated PC12 cells, neither ADA nor ZM241385 affected the basal [^{3}H]cAMP production (Fig. 3.35B). Also, ADA or ZM241385 did not affect the forskolin-stimulated or CGS21680-induced [^{3}H]cAMP production in NGF-treated cells (Fig. 3.35B).

ADA would not inhibit the binding of CGS21680 to A_{2A} receptors, so it was normal that chronic treatment with ADA would not affect the CGS21680-induced [^{3}H]cAMP production in untreated and NGF-treated PC12 cells. As the CGS21680-induced [^{3}H]cAMP production reflected the A_{2A} receptor expression in

PC12 cells, it was necessary to remove all the ZM241385 before the addition of CGS21680. Our preliminary data showed that in untreated PC12 cells incubated with ZM241385 for 2 days, the [^{3}H]cAMP production of cells washed twice (the same procedure in all cAMP assay) before the addition of CGS21680 was comparable to that in Fig. 3.27 under the same conditions (Fig. 3.35). Also, the CGS21680-induced [^{3}H]cAMP production of cells with four times washes was similar to cells with only twice washes (Fig. 3.36). Therefore, ZM241385 was probably absent during the cAMP assay. As the CGS21680 responses in all the three treatment groups were comparable to each other, it suggested that ADA or ZM241385 were unlikely to affect A_{2A} receptor responses (Fig. 3.35B).

3.4.3.2 Acute effect of ADA and ZM241385

In untreated PC12 cells, the preliminary data showed that ADA and ZM241385 appeared not to affect the basal [^{3}H]cAMP production, but seemed to increase the forskolin response slightly (Fig. 3.37A). The CGS21680 response was higher in ADA-treated group compared with control, while ZM241385 appeared to suppress the CGS21680-meidated [^{3}H]cAMP production (Fig. 3.37A).

In NGF-treated PC12 cells, the basal [^{3}H]cAMP production of control, ADA-treated or ZM241385-treated group were comparable to each other, while the

forskolin response was found to be higher in ADA- or ZM241385-treated groups (Fig. 3.37B). Similar as the findings obtained for the untreated PC12 cells, in the presence of ZM241385, the CGS21680-meidated [^{3}H]cAMP production was suppressed in NGF-treated PC12 cells (Fig. 3.37B).

As ZM241385 was present during the treatment with CGS21680, therefore, the CGS21680-induced [^{3}H]cAMP production was suppressed in untreated and NGF-treated PC12 cells under acute treatment of ZM241385. Also, ADA did not affect the binding of CGS21680 to A_{2A} receptors, therefore, acute treatment with ADA would not affect the CGS21680-induced [^{3}H]cAMP production in untreated and NGF-treated PC12 cells. However, it was unexpected that acute treatment with ADA or ZM241385 could increase forskolin-stimulated [^{3}H]cAMP production in NGF-treated PC12 cells, and it requires further study to clarify this point.

3.4.4 Discussion

In this part of study, we attempted to establish the relationship of A_{2A} receptors in the enhanced forskolin-stimulated [^{3}H]cAMP production in NGF-treated cells, although we did not get the complete picture, some of the findings were still noteworthy. We found that the A_{2A} receptor was not regulated by NGF in our PC12 cells, instead of being down-regulated as observed from other research group (Arslan

et al., 1997; Nie et al., 1999), which raised the consideration that our PC12 cells are not necessarily identical to others. Also, we confirmed the expression of A_{2A} receptor in our PC12 cells as ZM241385 significantly inhibited the CGS21680-induced [^{3}H]cAMP production, and the activation of A_{2A} receptor by CGS21680 and forskolin could give a synergistic activation on AC. Although there were some evidences showing that the activation of AC by forskolin was strongly dependent on A_{2A} receptor (Florio et al., 1999a; Florio et al., 1999b), the presence of activated A_{2A} receptor appeared not to be a prerequisite for the functioning of forskolin on AC in our PC12 cells. The enhanced forskolin-stimulated [^{3}H]cAMP production in NGF-treated PC12 cells on day 4 and day 6 was not related to the A_{2A} receptor, as there was no significant difference of A_{2A} receptor response between control and NGF-treated cells on day 4 and day 6. Also, in our preliminary study on the chronic and acute effect of ADA and ZM241385, there was no solid result showing that either ADA or ZM241385 could inhibit the forskolin response chronically or acutely.

In conclusion, we found that the enhanced forskolin-stimulated [^{3}H]cAMP production in NGF-treated cells (in section 3.2) was probably not due to the increase of A_{2A} receptor expression after prolonged NGF treatment, which strengthen our hypothesis that NGF promote gene and protein expression of AC during differentiation and lead to the increase of forskolin-stimulated [^{3}H]cAMP production.

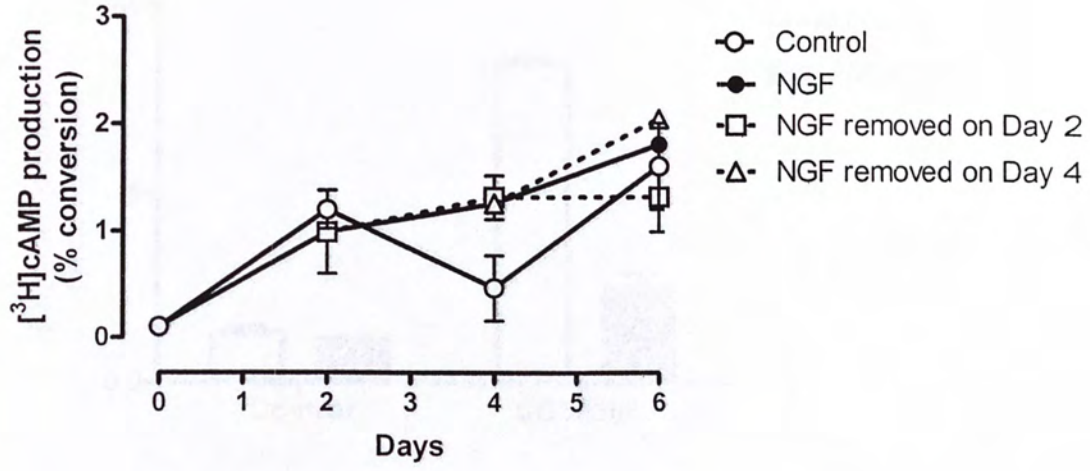


Fig. 3.31. The effect of NGF on CGS21680-induced [^{3}H]cAMP production by PC12 cells. PC12 cells were maintained in low-serum medium (± NGF, 50 ng/ml), and medium was renewed every 2 days. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with selective adenosine A_{2A} receptor agonist, CGS21680 (1 μM) for 30 min. Results represent mean ± S.E.M. of three independent experiments performed in duplicate.

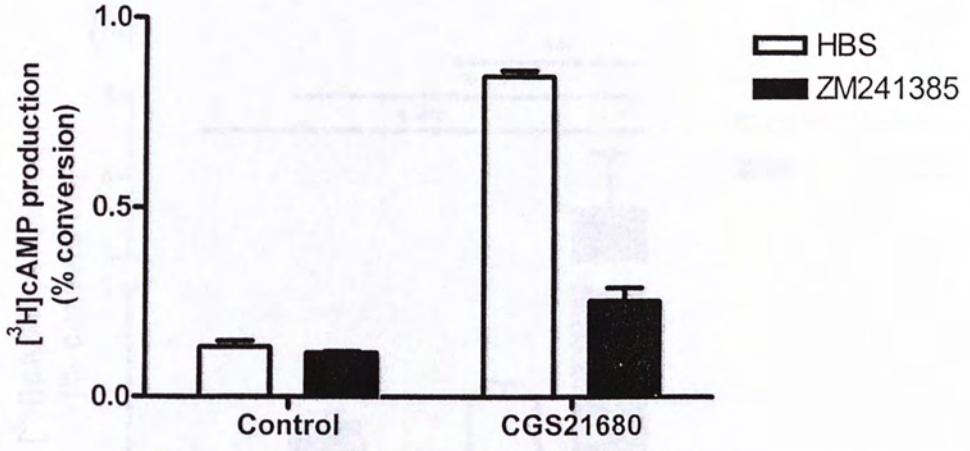


Fig. 3.32. ZM241385 (A_{2A} receptor antagonist) blocked CGS21680-mediated [^{3}H]cAMP production of PC12 cells. PC12 cells were maintained in low-serum medium for 2 days. After pre-labelling with [^{3}H]adenine, PC12 cells were pre-incubated with HBS or ZM241385 (1 μM) for 30 min. Then, cells were incubated with HBS or CGS21680 (1 μM) for another 30 min. Results represent mean ± S.E.M., from one experiment performed in triplicate.

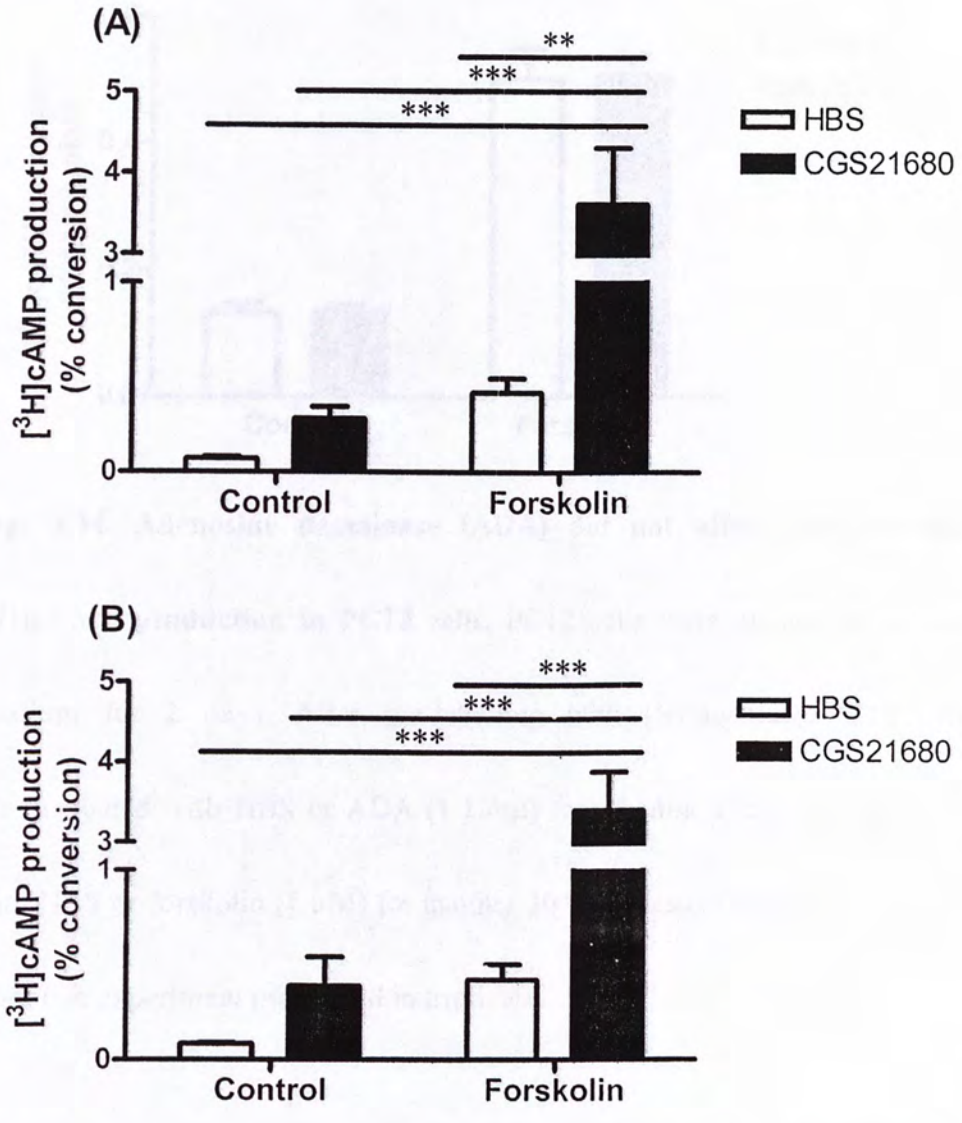


Fig. 3.33. CGS21680 and forskolin work synergistically to increase [^{3}H]cAMP production of PC12 cells. PC12 cells were maintained (A) in complete culture medium for 48 h or (B) treated with NGF for 32 h in low-serum medium. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with HBS or CGS21680 (1 μM) alone, or in the presence of forskolin (1 μM) for 30 min. Results represent mean ± S.E.M. of three independent experiments performed in triplicate. (**P<0.01, ***P<0.001, one-way ANOVA with Bonferroni post test.)

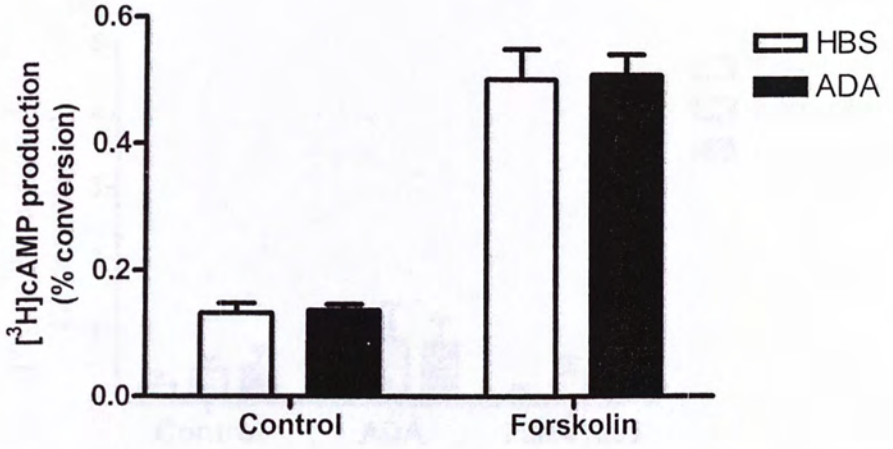


Fig. 3.34. Adenosine deaminase (ADA) did not affect forskolin-stimulated [^{3}H]cAMP production in PC12 cells. PC12 cells were maintained in low-serum medium for 2 days. After pre-labelling with [^{3}H]adenine, PC12 cells were pre-incubated with HBS or ADA (1 U/ml) for 30 min. Then, cells were incubated with HBS or forskolin (1 μM) for another 30 min. Results represent mean ± S.E.M., from one experiment performed in triplicate.

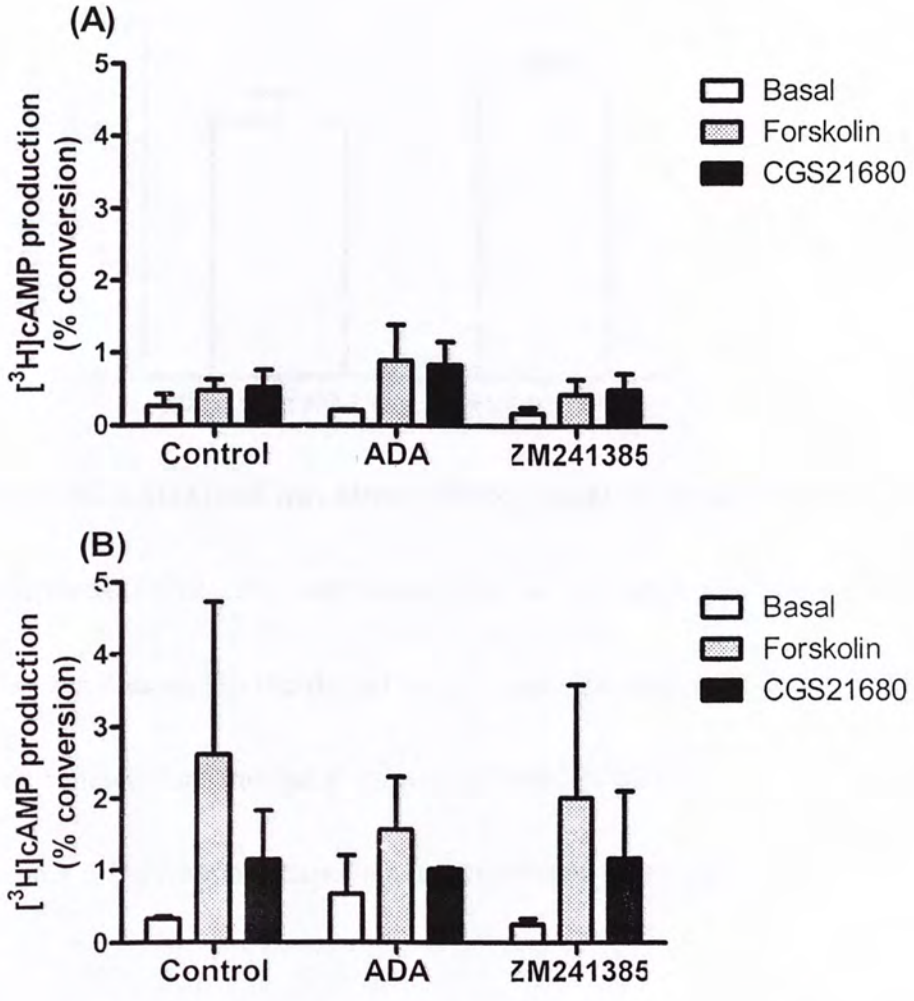


Fig. 3.35. The chronic effect of adenosine deaminase (ADA) or ZM241385 on [^{3}H]cAMP production of PC12 cells. PC12 cells were maintained in low-serum medium (A) in the absence (B) or presence of NGF for 4 days. ADA (1 U/ml) or ZM241385 (1 μM) were added 3 h before the addition of NGF on Day 0. Then, drugs and medium were renewed on day 2. After pre-labelling with [^{3}H]adenine, PC12 cells were incubated with HBS, forskolin (1 μM) or CGS21680 (1 μM) for 30 min. Results represent mean ± S.D. of two independent experiments performed in duplicate.

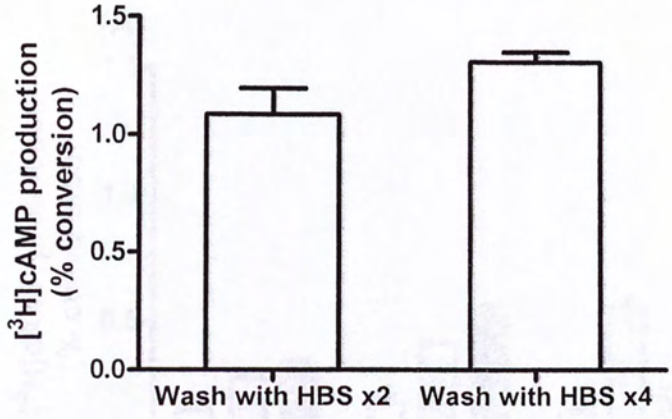


Fig. 3.36. ZM241385 was absent during cAMP assay of PC12 cells after chronic treatment. PC12 cells were maintained in low-serum medium with ZM241385 (1 μM) for 2 days. On the day of assay, cells were either washed with HBS twice or four times before incubated with CGS21680 (1 μM) for 30 min. Results represent mean ± S.D., from one experiment performed in duplicate.

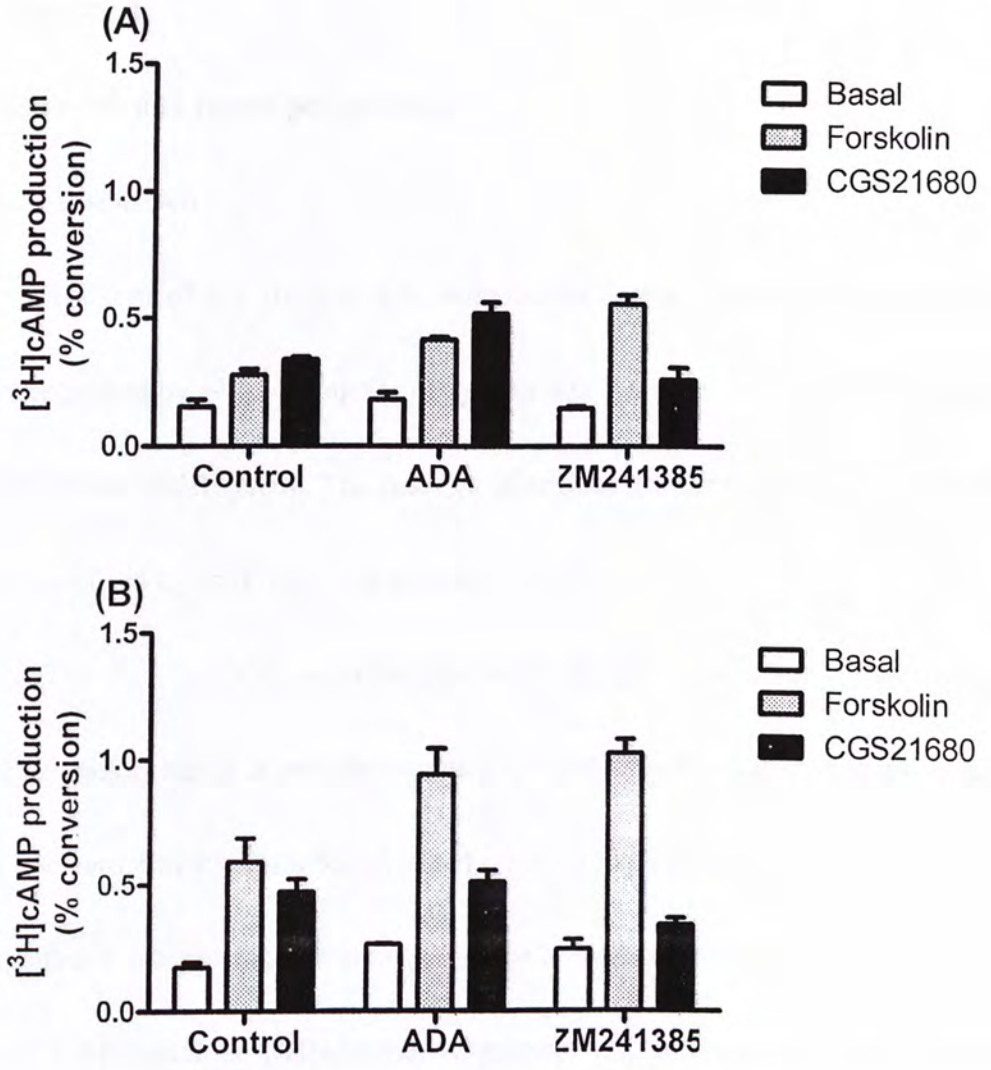


Fig. 3.37. The acute effect of adenosine deaminase (ADA) or ZM241385 on [^{3}H]cAMP production of PC12 cells. PC12 cells were maintained in low-serum medium (A) in the absence (B) or presence of NGF for 4 days. Drugs and medium were renewed on day 2. During the assay, cells were pre-treated with ADA (1 U/ml) or ZM241385 (1 μM) for 30 min. Then, cells were incubated with HBS, forskolin (1 μM) or CGS21680 (1 μM) for another 30 min. Results represent mean ± S.D., from one experiment performed in duplicate.

Chapter 4

Discussion and future perspectives

4.1 Discussion

The aim of this study was to examine the signal transduction pathways which are regulated by NGF during the early stages of neuronal differentiation using PC12 cells as the model system. The changes of cellular response and morphology of PC12 cells induced by NGF will be discussed.

The G_q-, G_s- or G_i- coupled prostanoid receptor expression in undifferentiated PC12 cells cultured in complete culture medium or cells treated with 50 ng/ml NGF in low-serum medium for 32 h were characterized by measuring the effect of various prostanoid receptor agonists on [^{3}H]IP or [^{3}H]cAMP production after prelabelling with [^{3}H]inositol or [^{3}H]adenine, respectively. Undifferentiated PC12 cells were unresponsive to all prostanoid receptor agonists tested for stimulation of PLC, revealed by the lack of stimulation in [^{3}H]IP production, which indicated that there were no G_q-coupled prostanoid receptors expressed in undifferentiated PC12 cells. Moreover, G_q-coupled prostanoid receptor expression was not induced by NGF in PC12 cells, as observed by the lack of prostanoid receptor agonist-stimulated [^{3}H]IP production in NGF-treated PC12 cells. In contrast, the positive controls, bradykinin and ATP, significantly increased [^{3}H]IP production in both undifferentiated and

NGF-treated PC12 cells through the G_q-coupled B_2 receptors and P2Y receptors, respectively. This result suggested that our experimental setup was feasible to detect [^{3}H]IP production, and the lack of response to prostanoid receptor agonists was not due to the absence of G_q proteins. Although TP receptor mRNA has been reported to be expressed in PC12 cells (Kitanaka et al., 1996), and the principal transduction of TP receptors was G_q, we found that the selective TP receptor agonist, U46619, did not stimulate [^{3}H]IP production in either undifferentiated or NGF-treated PC12 cells. There is no published data to suggest that TP receptors are functionally expressed in PC12 cells, and the presence of mRNA of TP does not necessarily imply the translation to TP receptor protein. Therefore, our findings are not contradictory to published literature as our PC12 cells are not necessarily identical to others. In summary, there were no G_q-coupled prostanoid receptors expressed in PC12 cells.

In addition, we did not find G_s-coupled prostanoid receptors expressed in PC12 cells, as no stimulation in [^{3}H]cAMP production was observed after treatment with various prostanoid receptor agonists. However, the failure for prostanoid receptor agonists to induce [^{3}H]cAMP production in PC12 cells was not due to the lack of G_s protein expression, because CGS21680 could significantly induce [^{3}H]cAMP production in undifferentiated or NGF-treated PC12 cells through the activation of G_s-coupled A_{2A} receptors. The G_i-coupled EP3 receptors were expressed in

undifferentiated PC12 cells, as three EP3 receptor agonists, PGE_2, sulprostone and ONO-AE-248, could suppress forskolin-stimulated [^{3}H]cAMP production, with PGE_2 having the highest potency. Also, PGE_2 no longer suppressed forskolin-stimulated [^{3}H]cAMP production in the presence of the EP3 receptor antagonist, ONO-AE3-240, which further proved the expression of G_i-coupled EP3 receptors in PC12 cells. The inhibition on forskolin-stimulated [^{3}H]cAMP production by the above EP3 receptor agonists appeared to be decreased after 32 h of NGF treatment, suggested that EP3 receptor expression might be down-regulated by NGF, however, we should not neglect the change of culturing condition from high- to low-serum state, which might also contribute to the change of EP3 receptor response. Although $PGF_{2\alpha}$ significantly inhibited forskolin-stimulated [^{3}H]cAMP production in undifferentiated PC12 cells, it was unlikely for the cells to express G_i-coupled FP receptors as FP receptor mRNA was found to be absent from PC12 cells (Kitanaka et al., 1996). Therefore, the $PGF_{2\alpha}$-induced response was probably due to the cross activation of EP3 receptors (Narumiya et al., 1999), as the $PGF_{2\alpha}$-induced response was decreased after NGF treatment, which follows the same pattern as the EP3 receptor agonists-induced response. As a result, after a comprehensive study on the prostanoid receptor expression in PC12 cells, we found that only EP3 receptors were expressed. However, we did not exclude the possibility that G_q- or G_s-coupled

prostanoid receptors might be expressed after NGF treatment longer than 32 h in PC12 cells.

The time course effect of NGF on EP3 receptor response of PC12 cells was studied. The change of EP3 receptor response over 6 days NGF treatment was determined by measuring the degree of PGE_2-mediated inhibition of forskolin-stimulated [^{3}H]cAMP production, which reflects the EP3 receptor expression in PC12 cells. We found that no matter PC12 cells were maintained in complete culture medium or low-serum medium, NGF appeared not to change the EP3 receptor response, but the forskolin-stimulated [^{3}H]cAMP production was significantly enhanced after 4 days NGF treatment. When comparing the basal or forskolin-stimulated [^{3}H]cAMP production between PC12 cells maintained in high- or low-serum condition, the responses for cells maintained in low-serum conditions was always lower than those in high-serum conditions. We speculated that the gene transcription responsible for cAMP production might be suppressed in low-serum conditions, so PC12 cells maintained in low-serum condition will have a lower basal and forskolin-stimulated [^{3}H]cAMP production. As a result, NGF did not influence the EP3 receptor response of PC12 cells over 6 days treatment, and the apparent down-regulation of EP3 receptor observed in section 3.1 was due to the transfer of cells from high- to low-serum condition. In order to confirmed that NGF really have

no effect on the regulation of EP3 receptor, it is better to perform radio-ligand receptor binding assay using [^{3}H]PGE_2, as this method can directly measuring the receptor expression level, but the cost of materials are much more expensive. It has been demonstrated that NGF increased COX-1 expression and endogenous PGE_2 production in PC12 cells (Kaplan et al., 1997), however, indomethacin did not block the neurite outgrowth in PC12 cells (DeGeorge et al., 1988). Together with our findings showing that NGF did not change the EP3 receptors expression in PC12 cells, the NGF-induced up-regulation of COX-1 and the subsequent production of prostanoids might have a paracrine instead of a autocrine role in PC12 cells.

NGF did not stimulate basal or enhance forskolin-stimulated [^{3}H]cAMP production in untreated or NGF-treated PC12 cells acutely, and the enhanced response to forskolin was maintained on day 6 when NGF was removed on day 4, but no persistent enhancement was observed at the later stage when NGF was removed on day 2, therefore, these results suggest that the enhanced forskolin response was most likely due to the chronic effect of NGF on gene and protein expression. NGF activates TrkA by phosphorylation which peaks at 5 min, and chronic treatment with NGF did not change the level of total or phosphorylated TrkA receptor level during the differentiation of PC12 cells (Chang et al., 2003b). Also, exposure of NGF-treated cells after 7 days to an additional NGF treatment did not

further increase the degree of activation of TrkA (Chang et al., 2003b). As we did not observe an increase in basal or enhance forskolin-stimulated [^{3}H]cAMP production in untreated or NGF-treated PC12 cells after exposure to NGF for 30 min, the activation of TrkA was not directly related to the [^{3}H]cAMP production.

Among AC2, AC6 and AC8, only AC6 was detected in our PC12 cells. However, the preliminary data showed that the AC6 expression was not up-regulated by NGF after 4 days or 6 days treatment, which did not match with the enhanced forskolin response observed on day 4 and day 6. Phosphorylation had been reported to inhibit the AC6 activity (Chern et al., 1995). Therefore, NGF might attenuate this inhibitory signal rather than up-regulate the AC6 expression to provide the enhanced forskolin response. Moreover, other AC isoforms might be responsible for the enhanced forskolin response in NGF-treated PC12 cells, so it is necessary to characterize all the AC isoforms in PC12 cells in order to establish the relationship between NGF and the enhanced forskolin-stimulated [^{3}H]cAMP production. We speculated that the maintenance of AC level in PC12 cells was NGF-independent at the later stage of differentiation, and if the elevation of cAMP production is directly related to differentiation, the change of cAMP production during the time course of NGF treatment should match with other differentiation markers, such as the expression of NfH and expression of neurites.

NGF is reported to promote the expression of NfH in PC12 cells in a time-dependent manner (Liu et al., 2006). Our preliminary data did not show an obvious dose- or time-dependent relationship between NGF and level of NfH expression. The NfH level was slightly increased after 1 day NGF treatment and than maintained steady to day 4, which might suggest that differentiation of PC12 cells was completed within 1 day NGF treatment. However, manual assessment or quantification of images of PC12 cells showed that NGF increased the proportion of cells expressing neurites in a time-dependent manner, which implied that the NfH expression should also increase in a similar pattern. As we did not observe an increase of NfH expression in PC12 cells exposed to NGF, it might be that our PC12 cells could express neurites independent of NfH or the experimental setup was not sensitive enough to detect the change of NfH expression.

As we failed to use NfH expression as the marker of differentiation of PC12 cells, we employed a neurite assay to quantify the degree of differentiation. We found that NGF can promote differentiation of PC12 cells by increasing the proportion of neurite-expressing cells, length of neurite, and the average number of neurite expression, and all these parameters decreased when NGF was removed on day 2 or day 4. Although the enhanced forskolin-stimulated [^{3}H]cAMP production was probably related to an increase of AC expression, which was maintained on day 6

when NGF was removed on day 4, this response was not enough or even not related to the maintenance of neurites. The cAMP analog, dibutyryl cAMP, has been reported to initiate neurite outgrowth by itself in PC12 cells, or act synergistically with NGF to promote neurite outgrowth (Gunning et al., 1981b; Heidemann et al., 1985; Hayakawa et al., 1999). Unlike NGF, dibutyryl cAMP initiates neurite outgrowth independent of RNA synthesis, and the dibutyryl cAMP-induced neurite expression was not maintained when dibutyryl cAMP was replaced by NGF (Gunning et al., 1981b). The dibutyryl cAMP induced neurite outgrowth was quite rapid (response occurred after a few minutes), while NGF required several days to induce neurite in PC12 cells (Heidemann et al., 1985). The rapid response of dibutyryl cAMP suggested that the neurite induced by dibutyryl cAMP was by reorganization of the existing materials for the cytoskeleton and was independent of new RNA or protein synthesis, while NGF induced neurite by new RNA and protein synthesis. The use of dibutyryl cAMP or cholera toxin, which increases cAMP level, could enhanced the NGF-induced differentiation in a synergistic manner in PC12 cells, revealed by the increase of neurite length and the proportion of cells with neurites (Gunning et al., 1981a; Heidemann et al., 1985), which suggested that cAMP and NGF induced neurite outgrowth through different mechanisms. Therefore, these published findings suggest that cAMP is involved in the initiation of neurite

outgrowth while NGF is required for the maintenance of neurites through the synthesis of RNA and protein and is independent of cAMP in PC12 cells.

Although in PC12 cells stably expressing EP3B receptor treated with EP3 receptor agonist can induce neurite retraction through a PTX-insensitive but Rho-sensitive pathway (Katoh et al., 1996; Katoh et al., 1998a; Aoki et al., 1999), either activation or blockade of EP3 receptors in our PC12 cells did not influence the neurite expression. These results suggested that EP3 receptors might not directly regulate neurite outgrowth in PC12 cells, or the strength of the signal of EP3 receptor response was not enough to trigger neurite retraction in non-transfected cells. The EP3 receptors might therefore not be essential for the development of the nervous system, because EP3 knockout mice are viable. However, EP3 receptors are required for the proper functioning of the nervous system, as EP3 knockout mice lost their hyperalgesia response (Minama et al., 2001).

In the final part of this study, we attempted to establish the relationship of G_s-coupled A_{2A} receptors in the enhanced forskolin-stimulated [^{3}H]cAMP production in NGF-treated cells, as activated G_s can enhance the activation of AC by forskolin (Insel & Ostrom, 2003), but we need to emphasis that most of the data came from preliminary studies only. We have proved that A_{2A} receptors are expressed in PC12 cells, because the A_{2A} receptor antagonist, ZM241385, could suppress [^{3}H]cAMP

production induced by CGS21680, an A_{2A} receptor agonist. Also, NGF did not regulate the A_{2A} receptor expression, which suggested that A_{2A} receptor was not directly related to the enhanced forskolin-stimulated [^{3}H]cAMP production in NGF-treated PC12 cells. Moreover, co-stimulation of PC12 cells with CGS21680 and forskolin did lead to a synergistic activation of AC, but forskolin-stimulated [^{3}H]cAMP production was not suppressed by ADA, which might suggest that the activation of AC by forskolin was not dependent on any basal A_{2A} receptor activity. In addition, our results did not clearly demonstrated that neither ADA or ZM241385 treatment could inhibit forskolin-stimulated [^{3}H]cAMP production in PC12 cells, either acutely or chronically. As a result, the overall summary of this preliminary study is we have no solid findings suggest that A_{2A} receptor activity is involved in forskolin-stimulated [^{3}H]cAMP production in PC12 cells.

In conclusion, we have investigated the effect of NGF on the differentiation of PC12 cells. We have demonstrated that NGF did not appear to regulate EP3 and A_{2A} receptor expression, but the forskolin-stimulated [^{3}H]cAMP production was enhanced at the later stage of differentiation possibly due to the up-regulation of AC expression during the differentiation process. The increase of AC expression was not involved in the maintenance of neurite expression, which suggested that other factors regulated by NGF participated in the differentiation of PC12 cells. Perhaps this study

can provide valuable findings in order to understand the complex process of neuronal cell differentiation.

4.2 Future perspectives

NGF promoted differentiation of PC12 cells as revealed by the expression of neurites, however, NGF enhanced forskolin-stimulated [^{3}H]cAMP production probably through the increase expression of AC, but this change is not directly related to the maintenance of neurites. We speculated that cAMP formation is involved in the initiation of neurite outgrowth but not required for their maintenance, although NGF did not significantly elevate the [^{3}H]cAMP production at early time point (e.g. from day 0 to day 2). Forskolin (stimulates AC directly) and pituitary adenylyl cyclase-activating peptide (stimulates AC through the activation of G_s-coupled PAC1 receptor) can stimulate neurite outgrowth in PC12 cells (Vaudry et al., 2002; Hernandez et al., 1995). However, our co-investigators have demonstrated that the AC inhibitor, SQ22536, did not inhibit the neurite length of NGF-treated PC12 cells after 2 days, suggesting that cAMP produced from tmAC is not the source for the NGF-induced initiation of neurite outgrowth. It is known that cAMP is produced from tmAC and sAC, therefore, it is a good approach to test if cAMP produced from sAC is also involved in the initiation of neurite outgrowth in PC12

cells as it is activated by NGF (Stessin et al., 2006), using a selective sAC inhibitor, KH7 (Hess at al., 2005). If KH7 can block the neurite outgrowth of PC12 cells treated with NGF, then cAMP produced from sAC is the principal source involved in the initiation of neurite outgrowth.

Although we have discussed that G_i-coupled FP receptors are unlikely to be expressed in PC12 cells, and it is probably that $PGF_{2\alpha}$ cross-activates G_i-coupled EP3 receptors, it is still necessary to confirm this statement by determining the ability of $PGF_{2\alpha}$ to inhibit forskolin-stimualted [^{3}H]cAMP production in the presence of EP3 receptors antagonist, ONO-AE3-240, or FP receptor antagonist, such as AL-8810 (Sharif et al., 2001). If $PGF_{2\alpha}$ does not inhibit forskolin-stimulated [^{3}H]cAMP production in the presence ONO-AE3-240, then $PGF_{2\alpha}$ is most probably cross-activates the G_i-coupled EP3 receptors in PC12 cells. If $PGF_{2\alpha}$ effect was not abolished by the presence of ONO-AE3-240, but abolished by AL-8810, then there are G_i-coupled FP receptors expressed in our PC12 cells.

Finally, we have to characterize all the nine AC isoforms in PC12 cells, and study how NGF regulates their expression. We speculate that some of the AC isoforms will be up-regulated by NGF or the inhibitory signal on the AC activity will be suppressed, which lead to the enhanced forskolin-stimulated [^{3}H]cAMP production in PC12 cells.

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